Characterisation of proteins

Stephan Uebel,
Microchemistry Core Facility, MPI of Biochemistry

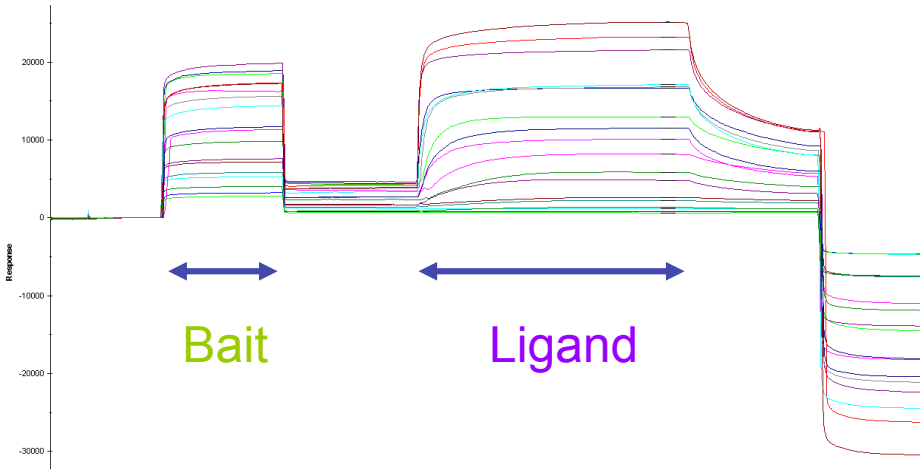
Proteincharacterization – Why?

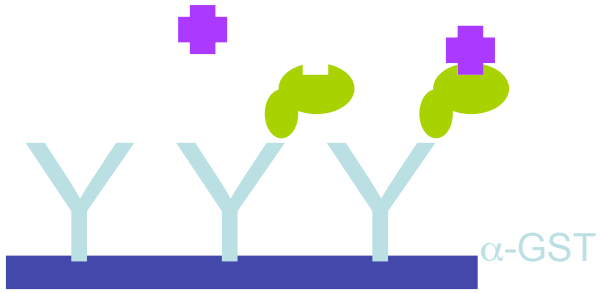
Proteincharacterization is done for peace of mind

The key to a good Itc experiment is buffer matching andprotein quality
Ernesto Frère @ DIPIA2010

Sample quality is key to interaction proteomics success
Ann-Claude Gingras, Mount Sinai Hospital

What are the requirements for performing high quality quantitative SPR experiments? 1. Learn how to recognize good quality data and 2. Prepare high quality samples, i.e. monodisperse and free of aggregates)
Eileen M. Lafer, UTHSCSA Center for Macromolecular Interactions



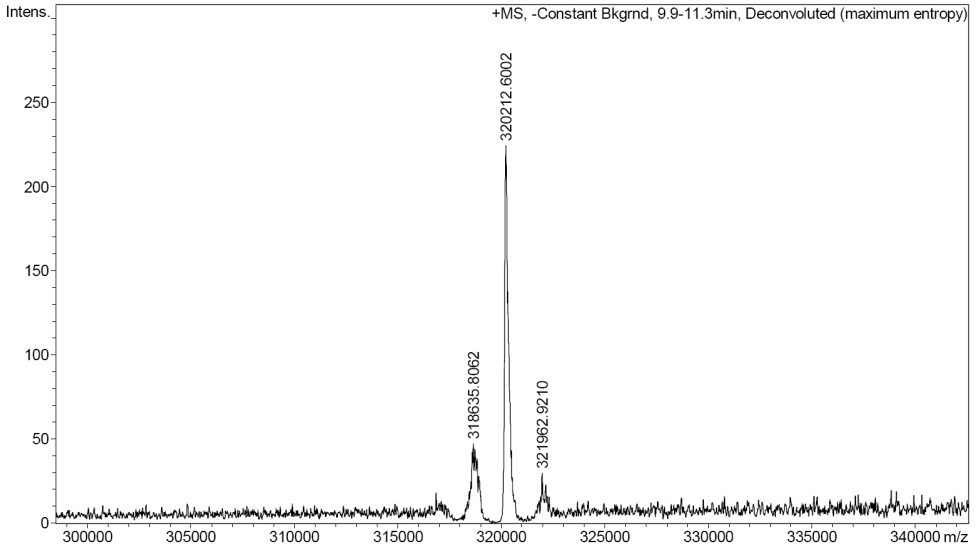


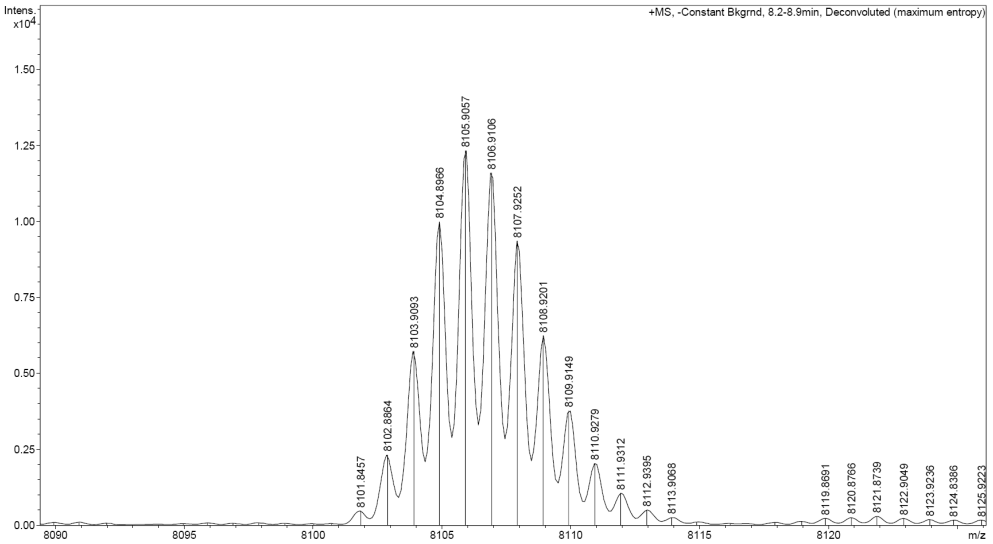
What to Characterize and How?

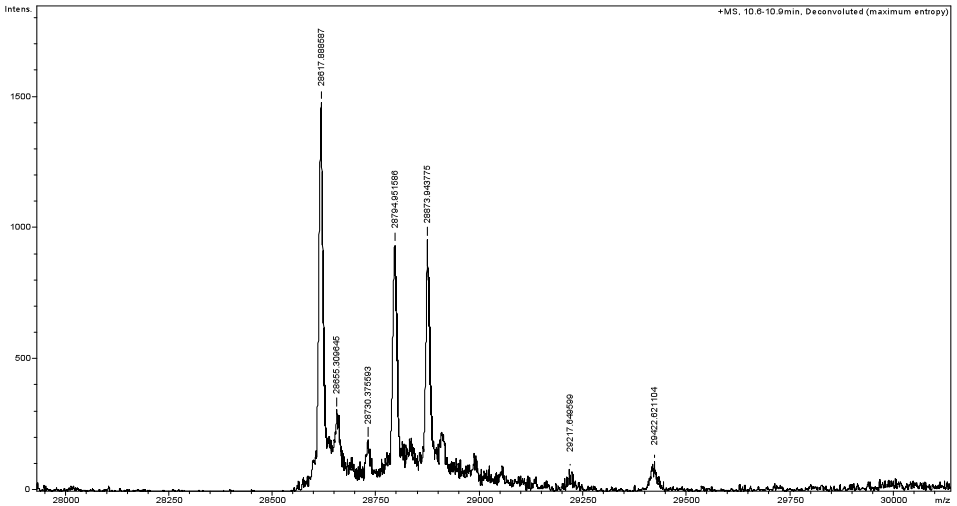
- Identitiy i.e. primary structure: amino acid sequence and desired posttranslational modifications
  - Edman sequencing combined with protein total mass (ESI-TOF, ESI-FTMS, [MALDI(IR)-TOF], deglycosylation of secreted proteins from insect cells and *S.cerevisiae*)
  - [peptide mapping by MS of tryptic digests]

Resolution in Total Protein MS

- LC-MS on C4 column with MeCN/water (0.05% TFA)







Analytical Biochemistry **267**, 169–184 (1999)
Article ID abio.1998.2990, available online at http://www.idealibrary.com on IDEAL®

Spontaneous α-*N*-6-Phosphogluconoylation of a "His Tag" in *Escherichia coli:* The Cause of Extra Mass of 258 or 178 Da in Fusion Proteins

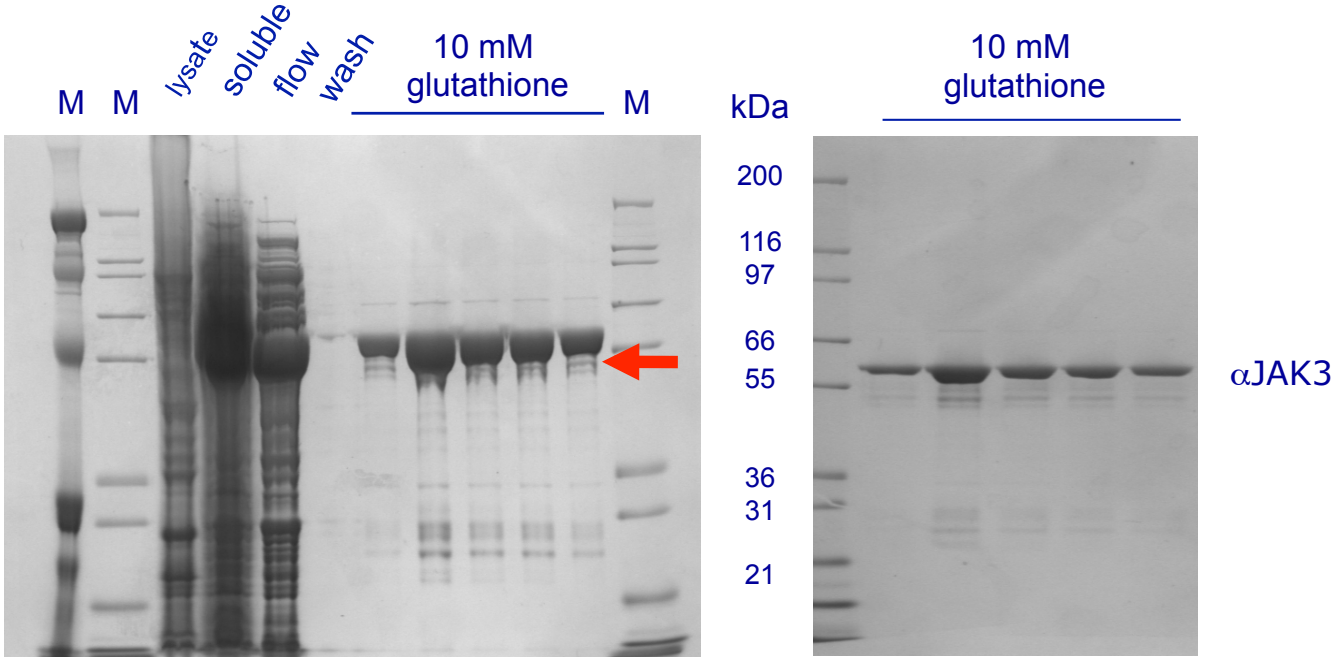
Kieran F. Geoghegan,*,[1] Henry B. F. Dixon,† Philip J. Rosner,* Lise R. Hoth,* Anthony J. Lanzetti,* Kris A. Borzilleri,* Eric S. Marr,* Linda H. Pezzullo,* LeRoy B. Martin,‡ Peter K. LeMotte,* Alexander S. McColl,* Ajith V. Kamath,* and Justin G. Stroh*

**Central Research Division, Pfizer Inc., Eastern Point Road, Groton, Connecticut 06340; †Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, United Kingdom; and ‡Micromass Inc., 100 Cummings Center, Suite 407N, Beverly, Massachusetts 01915*

Tyrosine Kinase JAK3

- Kinase domain expressed as ca. 60 kDa GST-fusion protein in *E. coli*:

- One-step purification on immobilized glutathione
- Analytical SEC and DLS: homogenous, ca. 800 kDa
- SDS-PAGE and immunoblotting:



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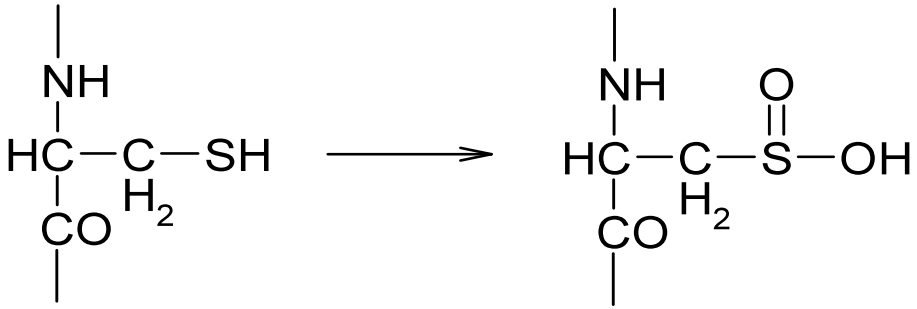
  - [peptide mapping by MS of tryptic digests]
- Secondary + tertiary structure
  - CD, FTIR
  - Thermal unfolding (DSC, CD, ThermoFluor® Assay)
  - Specific activity of enzymes, [ITC, CFCA Assay on SPR]

Cysteine Proteinase Z

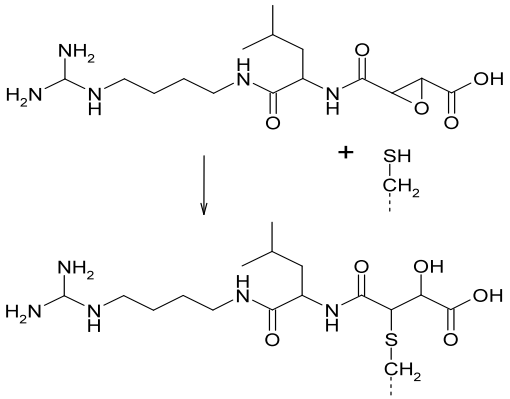
- 55 kDa proenzyme refolded from *E. coli* inclusion bodies, using factorial design
- Acid-induced autocatalytic cleavage and isolation by cation exchange chromatography
- Homogenous by SDS page
- Oxidation of active-site cysteine detected by X-ray crystallography

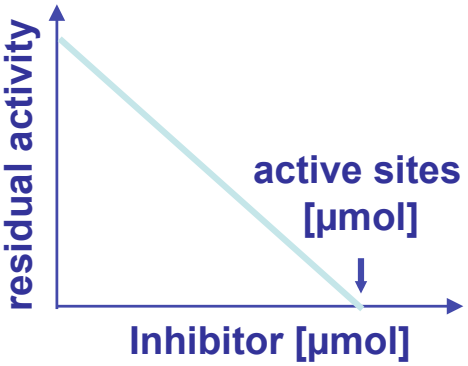


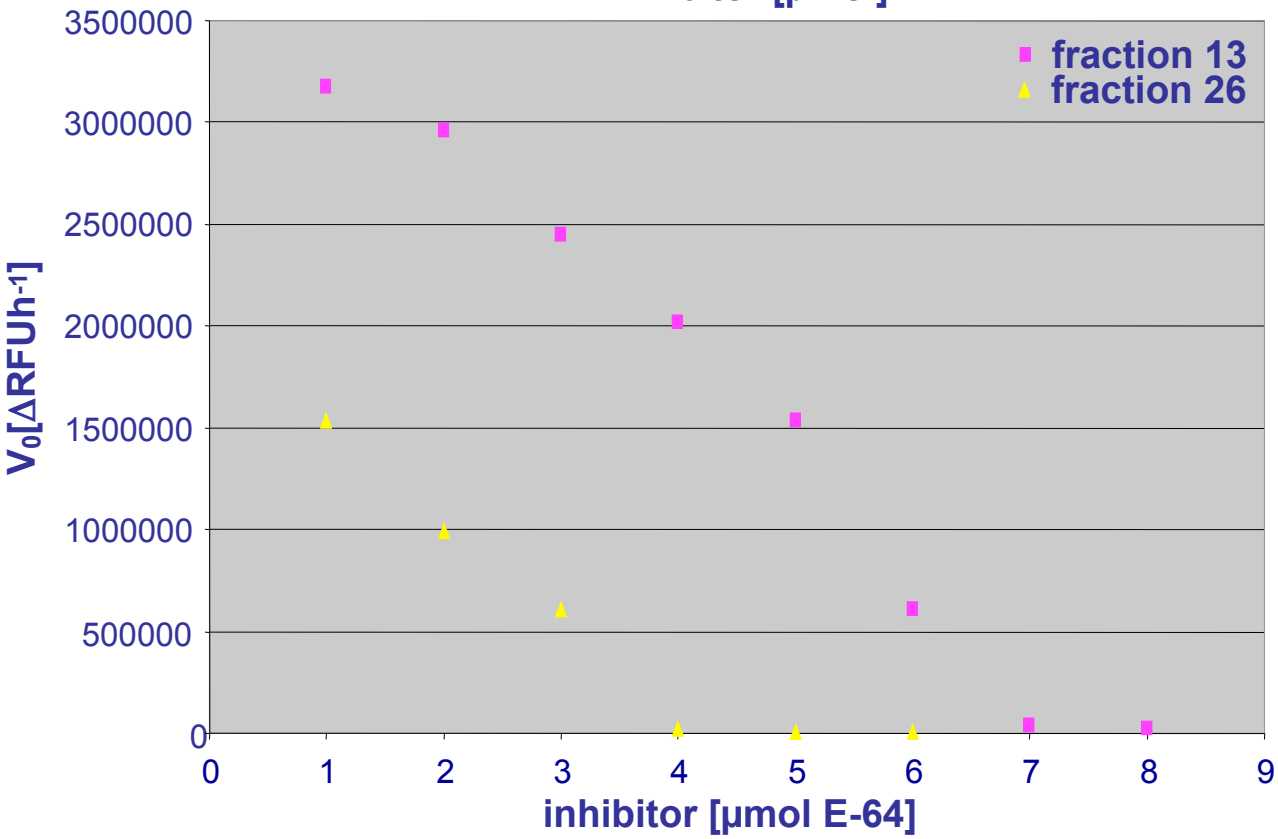
pK_s = 8-9 pK_s = 3-4

Active Site Titration

- E-64 is a covalent inhibitor for thiol proteases







SPR/Biacore-based methods

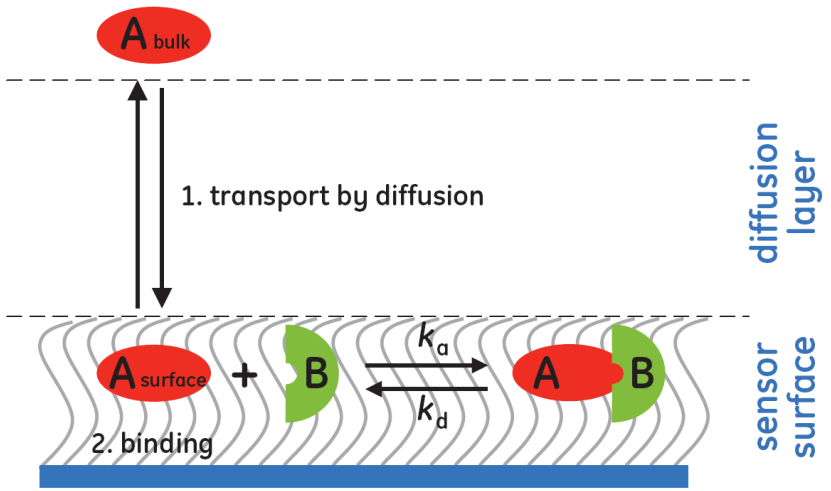
Example for an SPR-based assay : Calibration Free Concentration Assay CFCA

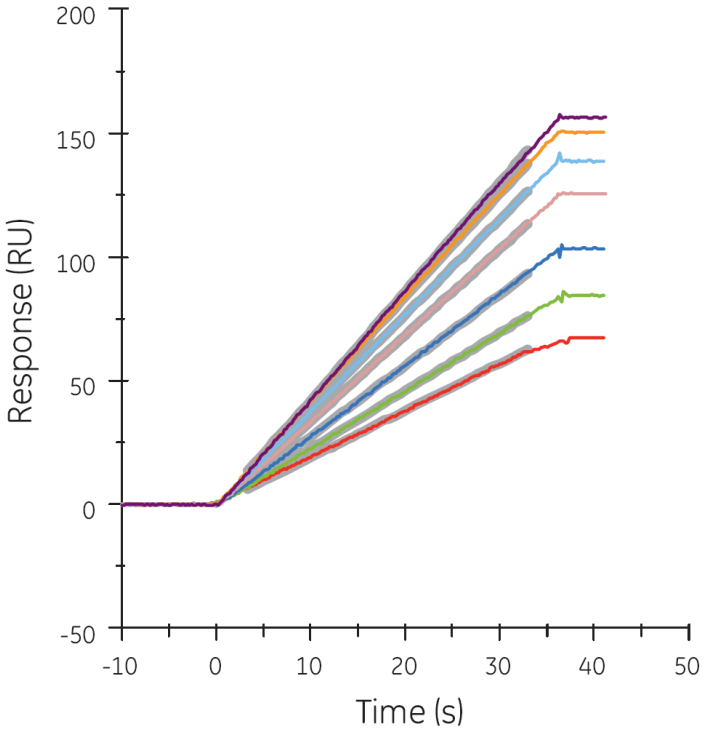
A_{bulk} is the concentration of injected analyte

$A_{surface}$ is the concentration of analyte at the sensor surface

B is the target immobilized on the surface

k_a and k_d are association and dissociation rate constants, respectively





Taken from: *Assessment of protein concentration using a calibration-free method*, K. Lindquist *et al.*, 2009, GE Healthcare publication BR-9100-252

→ disadvantage: specific antibodie(s) needed

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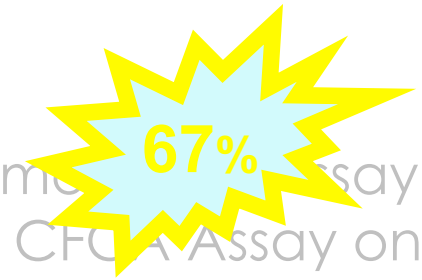
- Quarternary Structure: Oligomerization state + aggregation
  - DLS, SLS, SEC-MALS, AUC [UV, fluorescence, dye staining] etc.



What is aggregation?

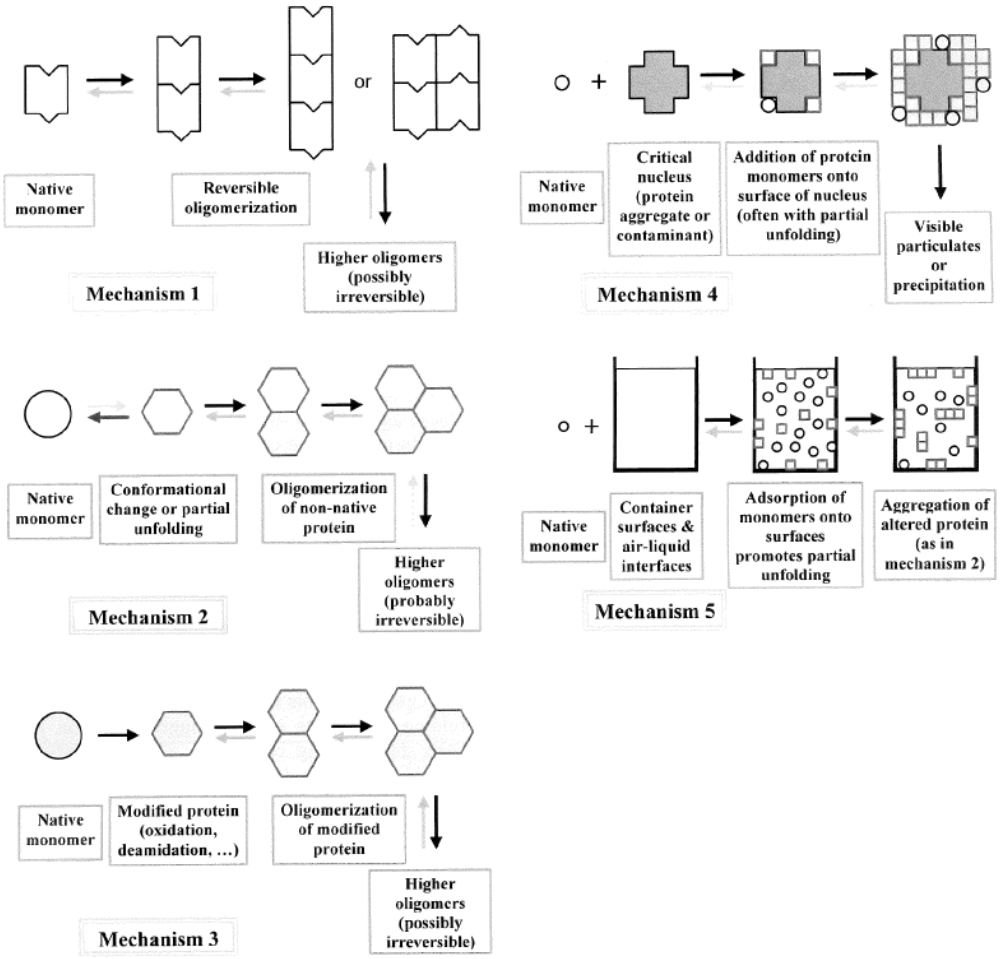
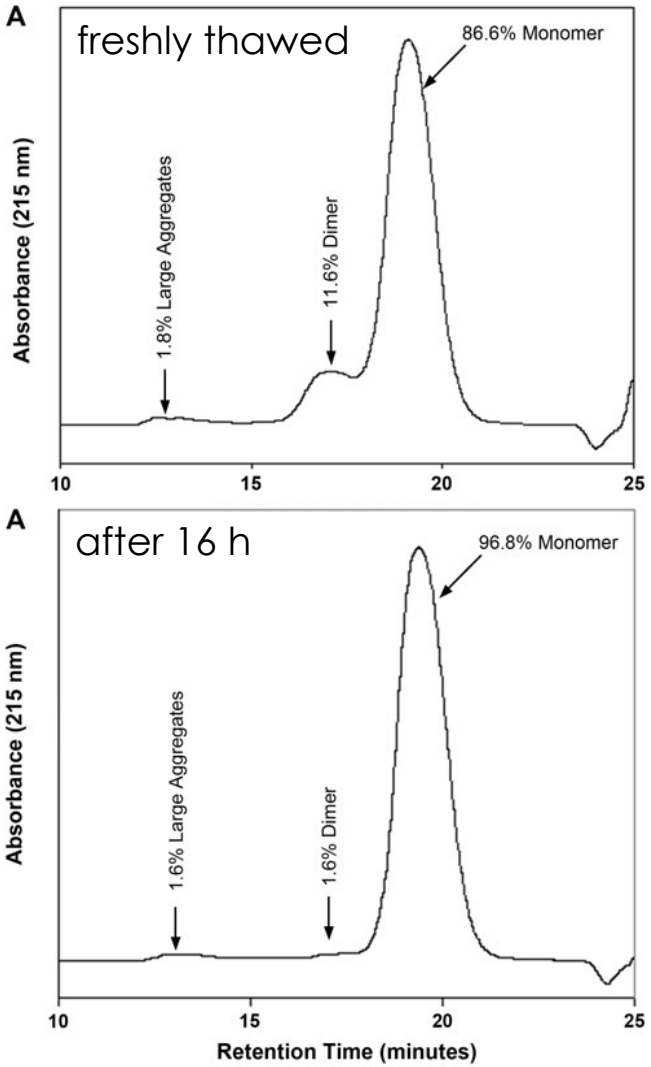


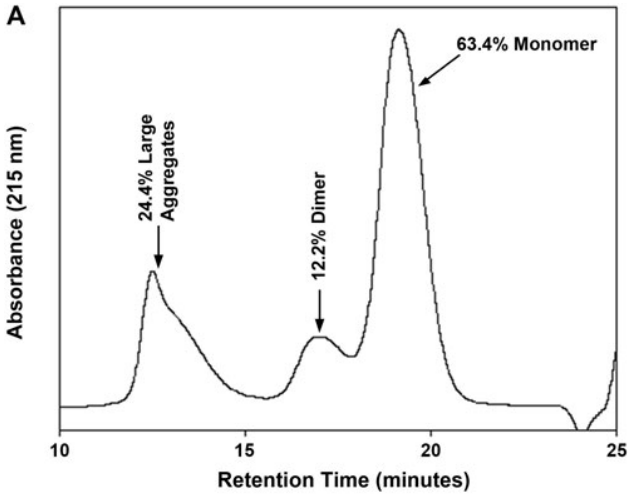
Fig. (1). Shematic illustrations of five common aggregation mechanisms.

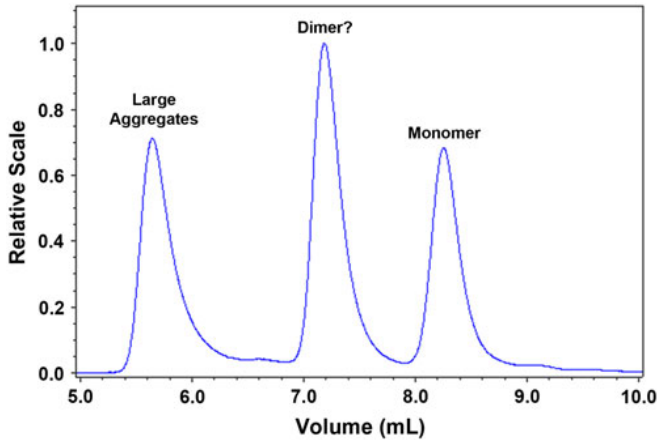
Taken from: Is any measurement method optimal for all aggregate sizes and types? John Philo, AAPS Journal 2006, E564ff.

But the most neglected aspect of aggregation is **kinetics!**



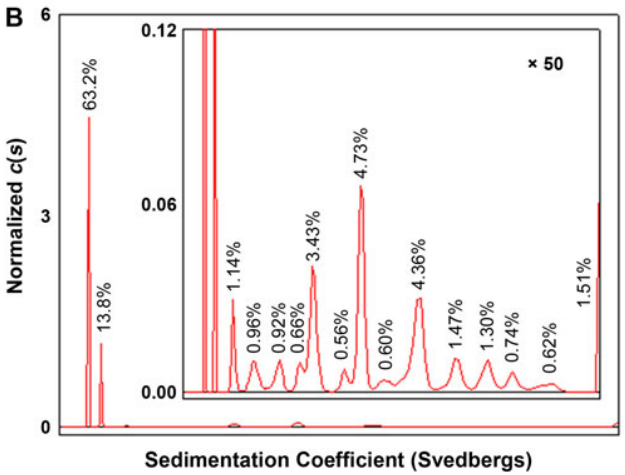
Usefullness of SEC as standard

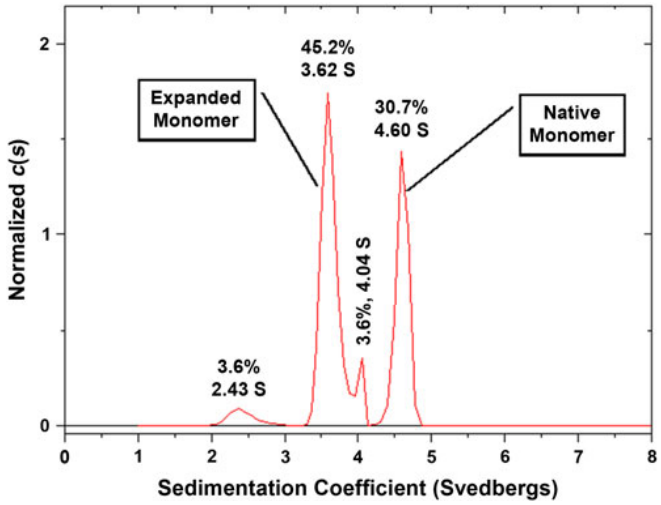




Dissociation or loss of aggregates can be caused by:	SEC	SV	FFFF
dilution	+++	+	+++
change of solvent conditions	+++	–	++
adsorption to surfaces	+++	+	++
physical filtration (eg, column frit)	+++	–	–
physical disruption (eg, shear forces)	++	–	–
Creation of new aggregates can be caused by:			
change of solvent conditions	+++	–	++
surface or shear-induced denaturation	++	–	+
concentration on surface	–	–	+

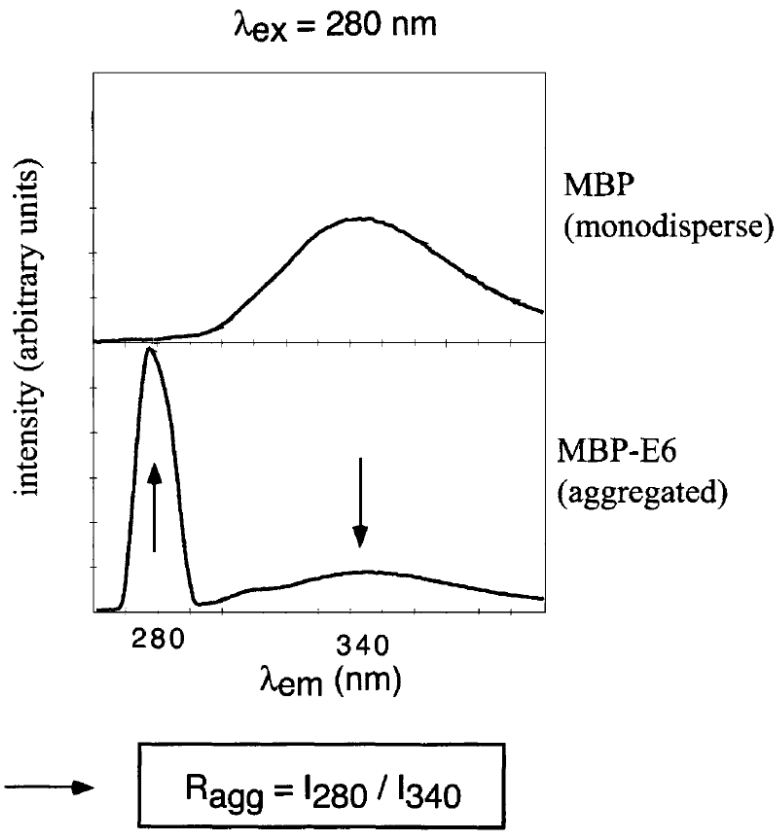
*The number of pluses indicates the relative size of the problem for that method.
SEC indicates size exclusion chromatography; SV, sedimentation velocity; FFFF, flow field-flow fractionation.





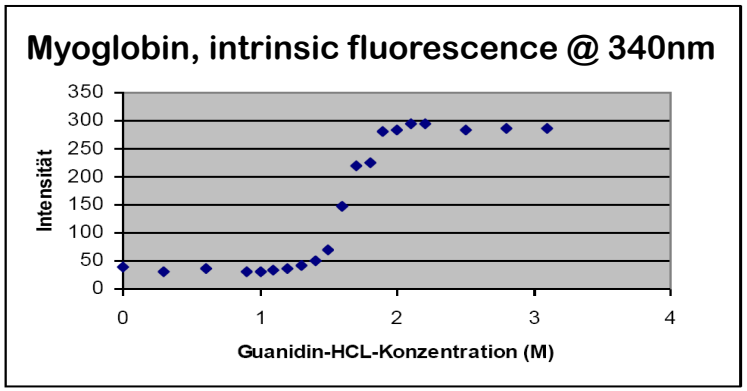
Usefullness of UV and Fluorescence

Aggregation index

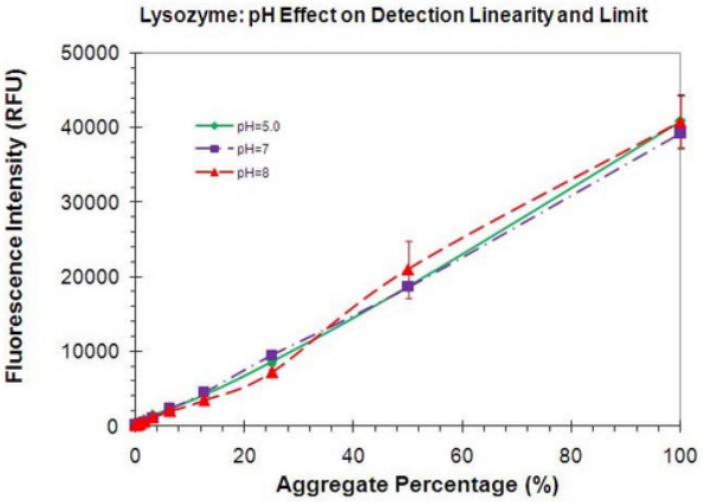


Nominé *et al.*, Protein Engineering 2006, 297ff.

But what about quantum efficiency?



Keller, Uni Graz



from: www.enzolifesciences.com

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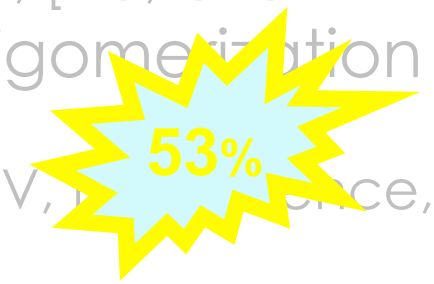
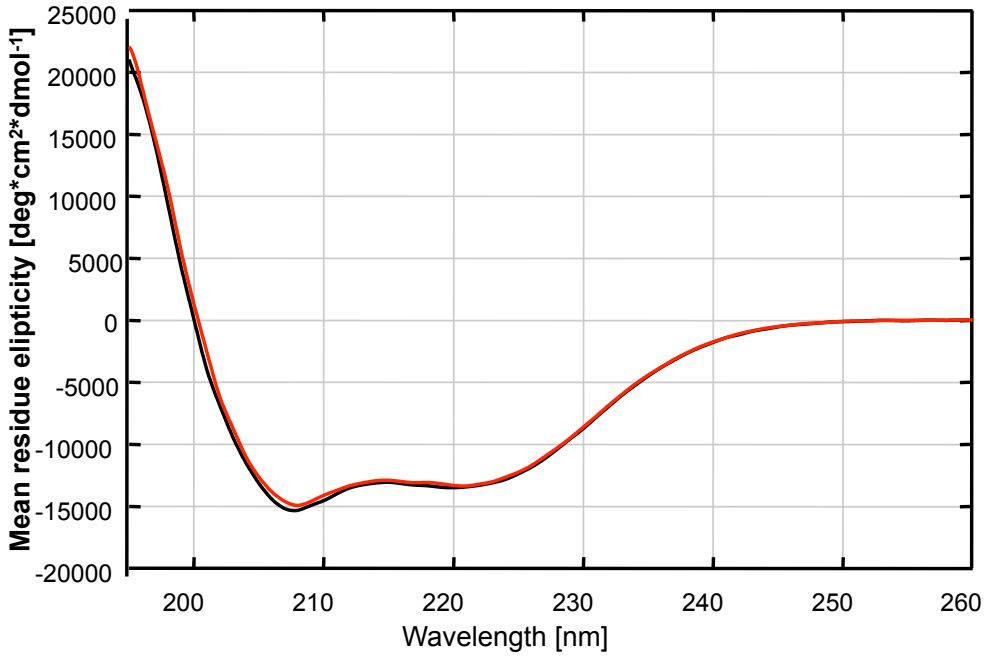
- Protein stability
  - Aggregation (and activity) as surrogate markers of stability



Matrix Metalloproteinase 12

- Protein stability investigated using CD spectroscopy:



	t=0	t=3 months
α-helix	38	38
B−sheet	14	13
turn	18	19
coil	29	30

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- Purity
  - *Cave*: lack of linearity in staining

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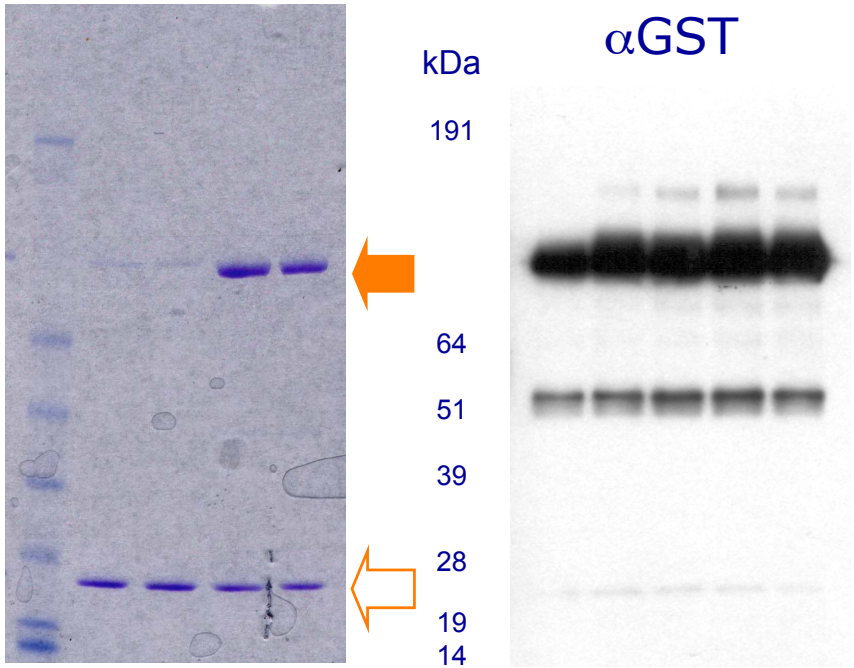
- Purity
  - *Cave*: lack of linearity in staining

Tyrosine Kinase BTK

- Kinase domain expressed as ca. 78 kDa GST- fusion protein in insect cells
- One-step purification on immobilized glutathione
- Contaminant at ca. 25 kDa recognised by αGST antibody, but not identified by MALDI-TOF MS

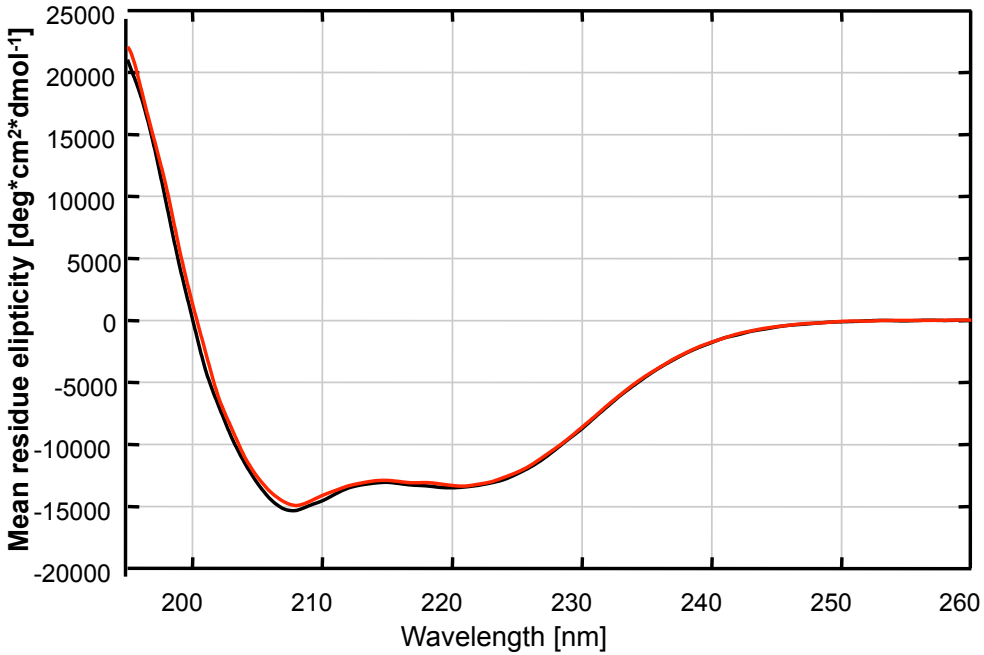


	E. coli		insect	
His_6	FL	KD	FL	KD
GST	FL	KD	FL	KD
MBP	FL	KD	FL	KD

Matrix Metalloproteinase 12

- Protein stability is investigated using CD spectroscopy and fluorescent dye binding

Far UV CD spectroscopy



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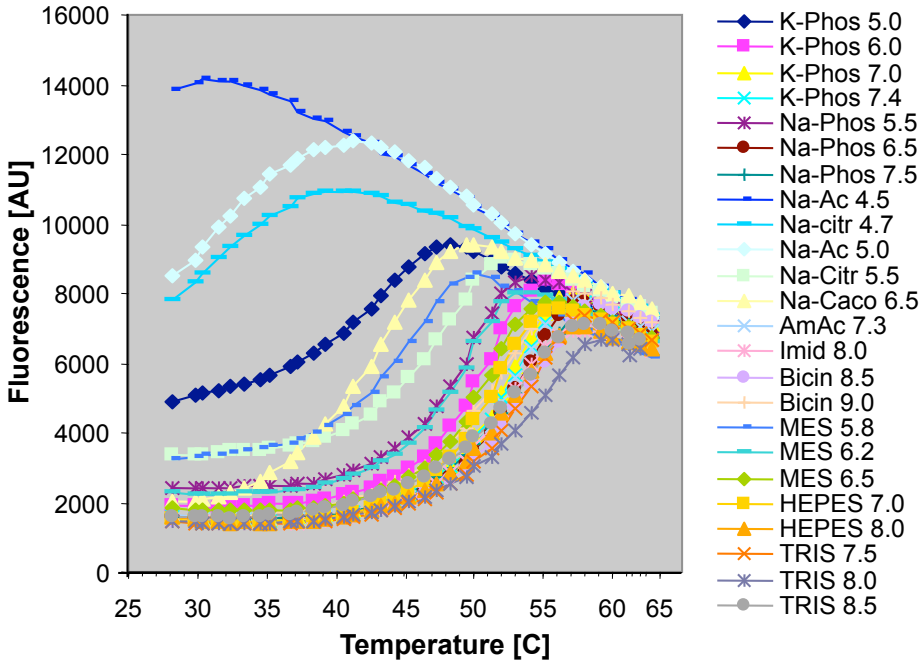
Thermal denaturation + Sypro Red



Overview

Peptide Chemistry
- Peptide synthesis
- Edman sequencing
- Amino acid analysis

1 FTEs

Light microscopy:
- Leica confocal mic
- RT deconvolutionv mic
- Spinning disc mic

1 FTE

Biophysics:
- Analyt. ultracentrifugation
- DLS, - Biacore
- CD , - FTIR, - Calorimetry

2 FTE

Protein Production:
- 4 expression systems
- Fermentation
- Purification + characteris.

5 FTEs

DNA Sequencing:
- Plasmids/PCR products
- Ready-to-load samples
- (O/N cultures)

1 FTE

RNAi:
- Synthesis of ribooligoribonucleotides
- shRNA library (h.+ m.)

1 FTEs

Mass Spectrometry:
- ESI-TOF
- Orbitrap

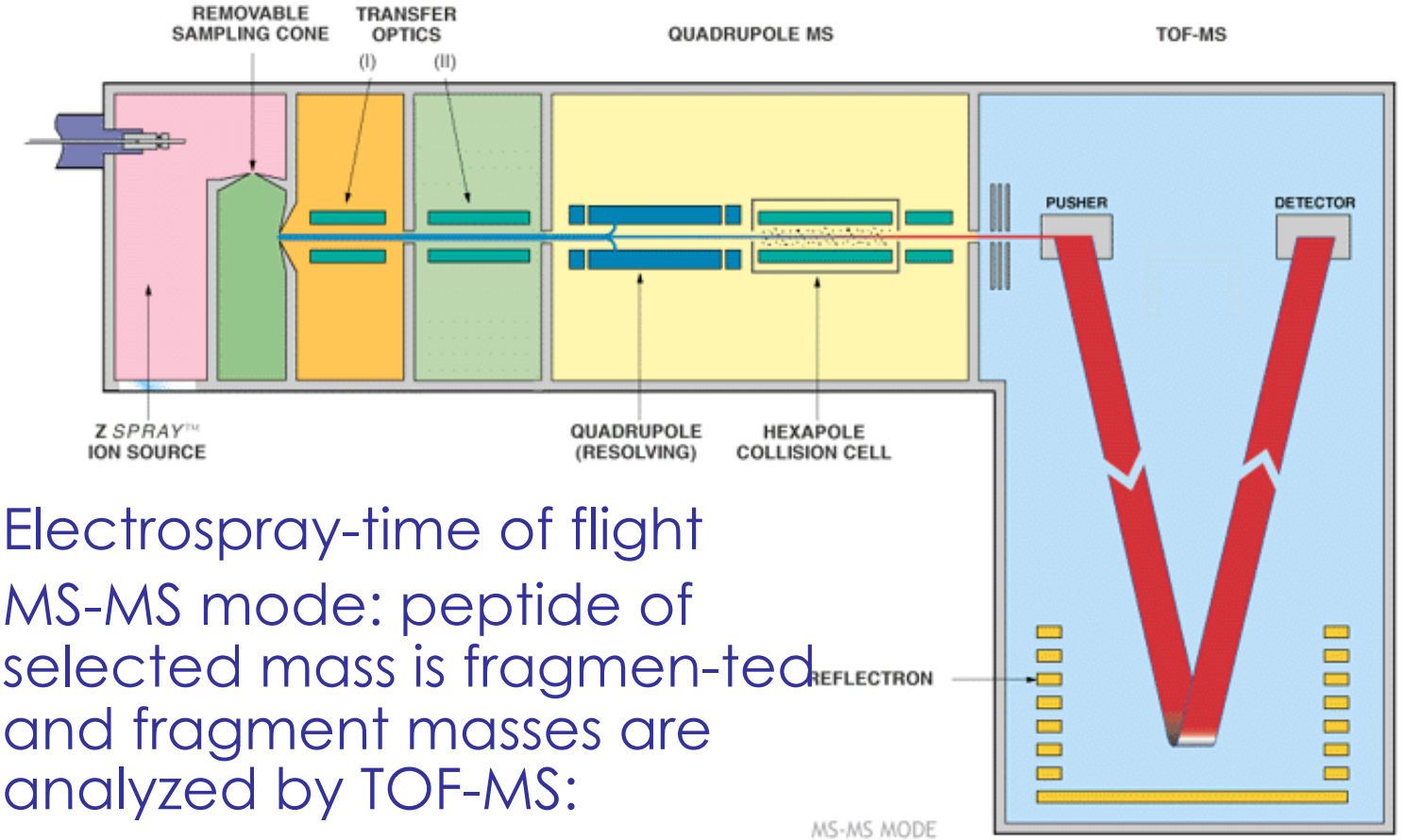
2 FTEs

Organic Chemistry:
-Synthesis of organic small molecules

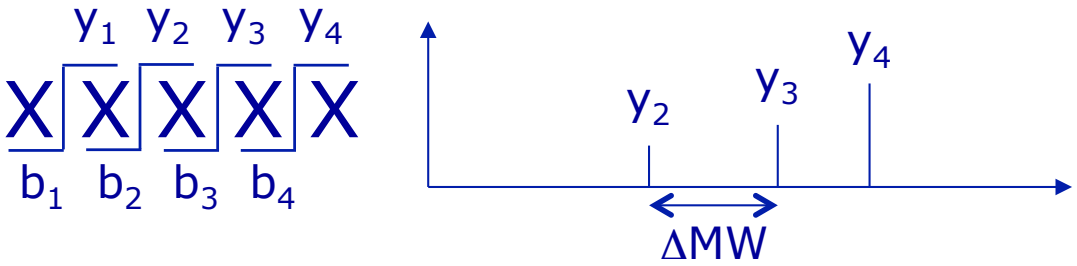
2 FTEs

Total: 16 full time employees (five scientists); open access instruments in orange

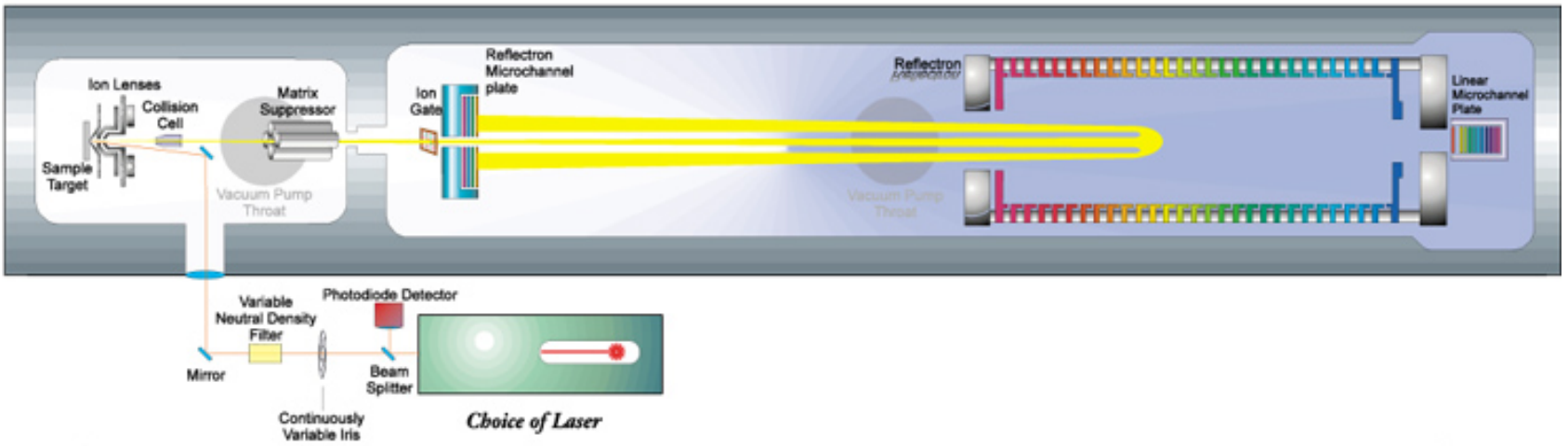
ESI-TOF Tandem MS MS



- Electrospray-time of flight
- MS-MS mode: peptide of selected mass is fragmen-ted and fragment masses are analyzed by TOF-MS:



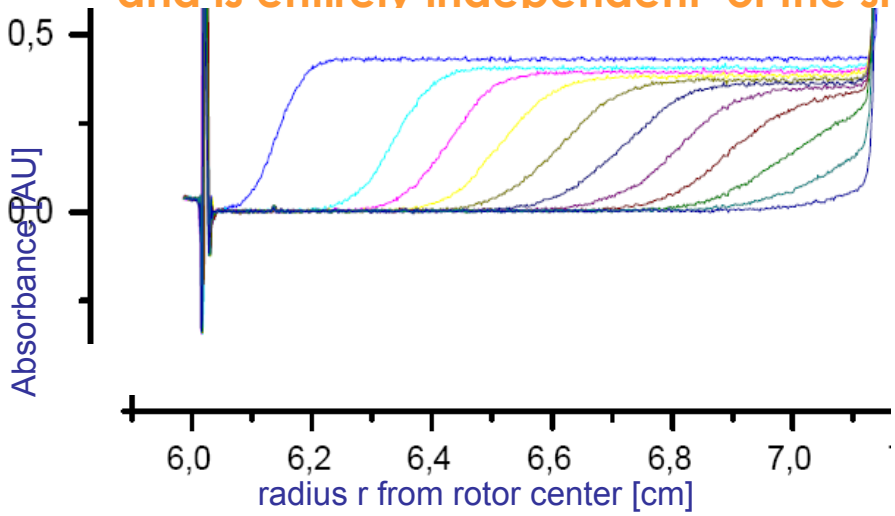
MALDI- TOF MS of Tryptic Digests

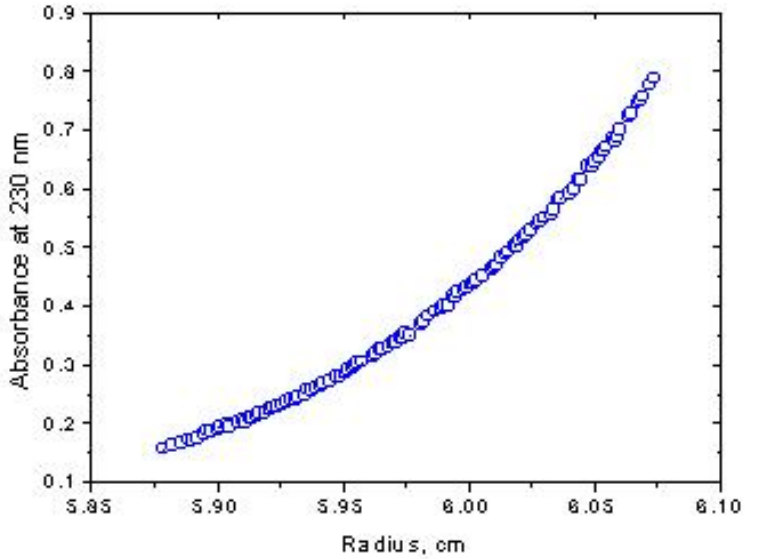


- Matrix assisted laser desorption ionization – time of flight mass spectrometry
- Peptides are embedded in a UV-absorbing matrix
- The list of peptide masses is run against a theoretical list of peptide masses after tryptic digest of proteins in the database of choice (e.g. SWISSPROT)

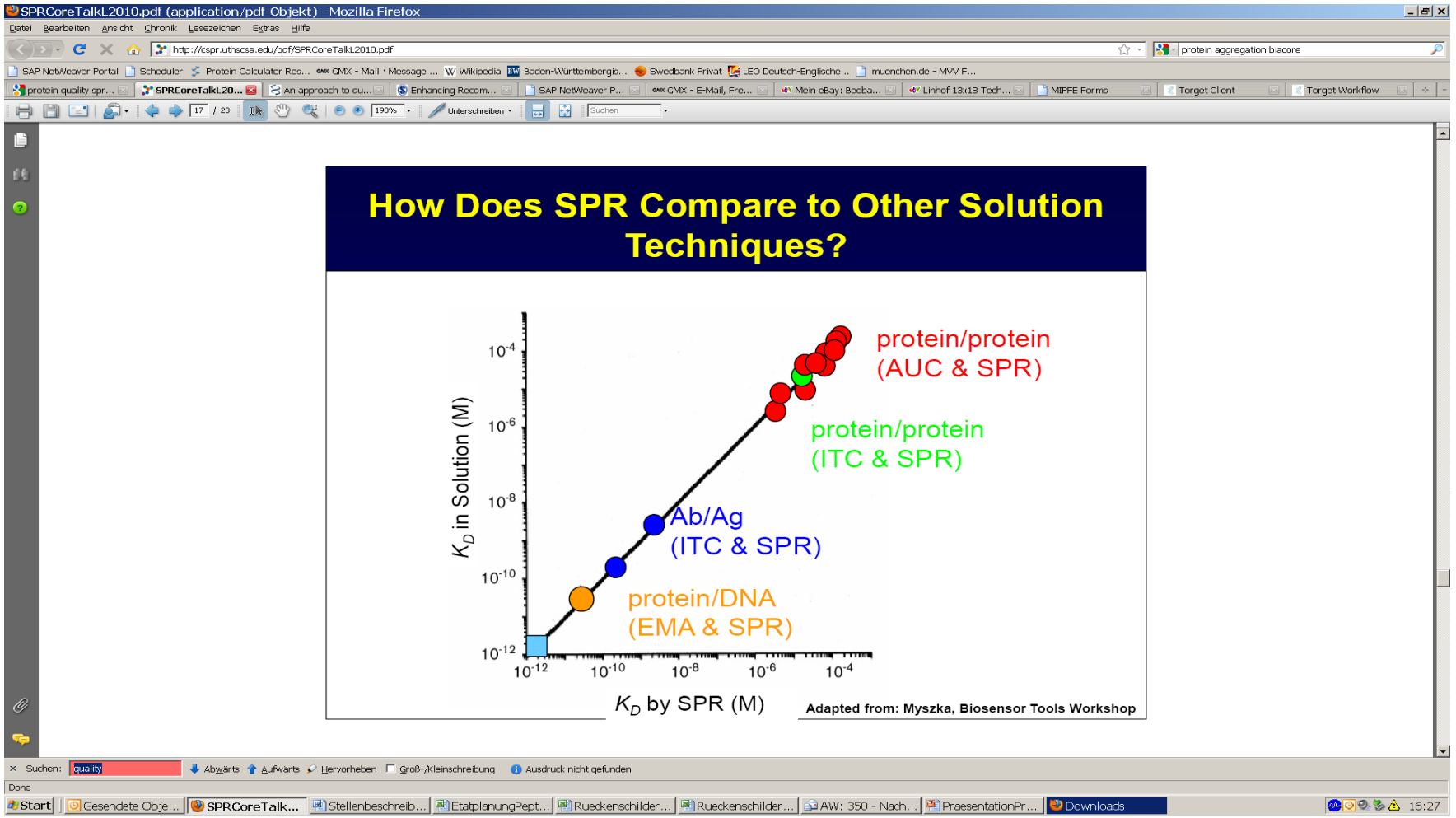
Analytische UZ

- **Sedimentation velocity** is an analytical ultracentrifugation method that measures the **rate at which the sedimentation boundary moves** in response to centrifugal force. It measures the **sedimen-tation coefficient (S)** which depends on the **molecular weight** (larger proteins sediment faster) and also on **molecular shape**.
- **Sedimentation equilibrium** measurements provide thermo-dynamically **rigorous, shape independent, molecular weights:**
  - As the centrifugal force produces a gradient in protein concentration across the centrifuge cell, diffusion acts to oppose this concentration gradient. Eventually, an exact balance is reached between sedimentation and diffusion, and the concentration distribution reaches an equilibrium. The concentration distribution at equilibrium **depends only on molecular mass, and is entirely independent of the shape of the molecule**.





Stephan


SPRCoreTalkL2010.pdf (application/pdf-Objekt) - Mozilla Firefox
How Does SPR Compare to Other Solution Techniques?
protein/protein (AUC & SPR)
protein/protein (ITC & SPR)
Ab/Ag (ITC & SPR)
protein/DNA (EMA & SPR)
K_D in Solution (M)
K_D by SPR (M)
10^{-4}
10^{-6}
10^{-8}
10^{-10}
10^{-12}
Adapted from: Myszka, Biosensor Tools Workshop