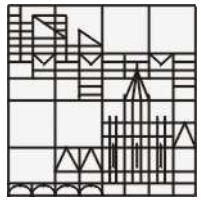
INNOMOL Workshop Proteomics
Ruder Boskovic Institute Zagreb
7th April 2014



Uncovering molecular details of protein "misfolding- aggregation" using affinity- and ion mobility- mass spectrometry: Physiological and "Parkinson"- Synucleins

Michael Przybylski

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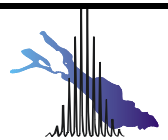
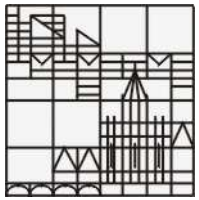
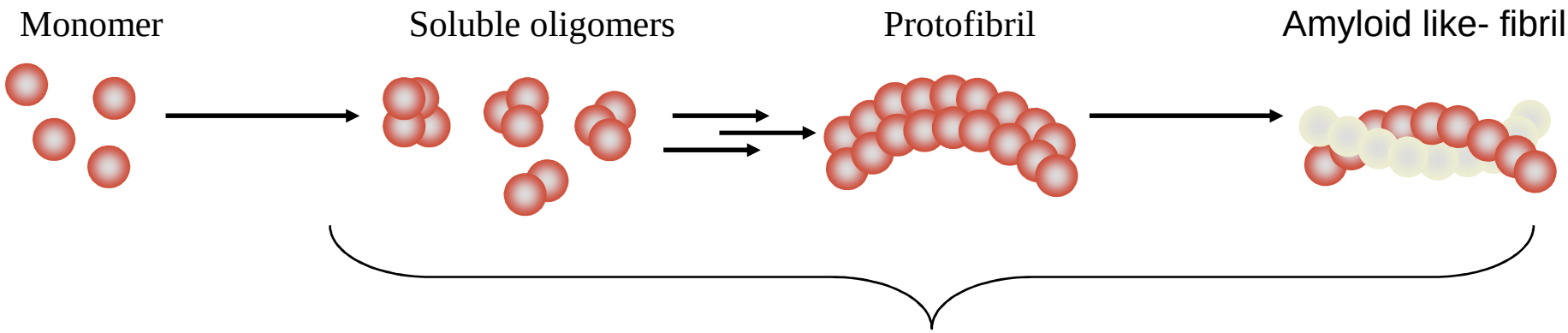
www.uni-konstanz.de/agprzybylski/chemie

www.affinityms.de



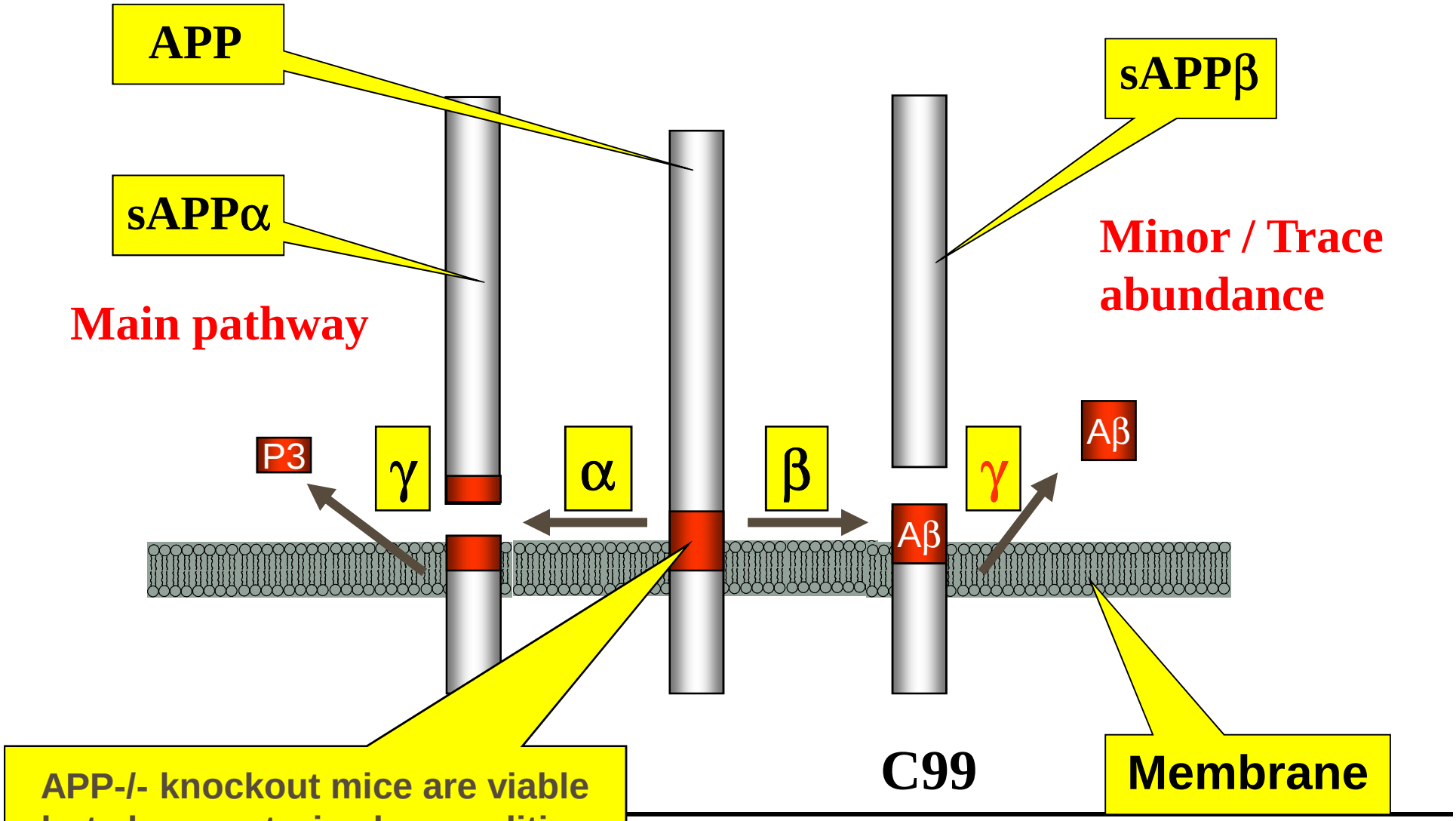
Protein „misfolding – aggregation - neurodegeneration -
Structural basis/mechanism of oligomerization?





Aß	**Alzheimer**
Tau	**Alzheimer**
αSynuclein	**Parkinson**
Prion	**Prion**
Huntingtin	**Huntington**

Key protein for “misfolding & aggregation”: APP (Amyloid Precursor Protein) or Aß?



APP-/- knockout mice are viable but show motoric abnormalities

Analytical Chemistry & Biopolymer Structure Analysis
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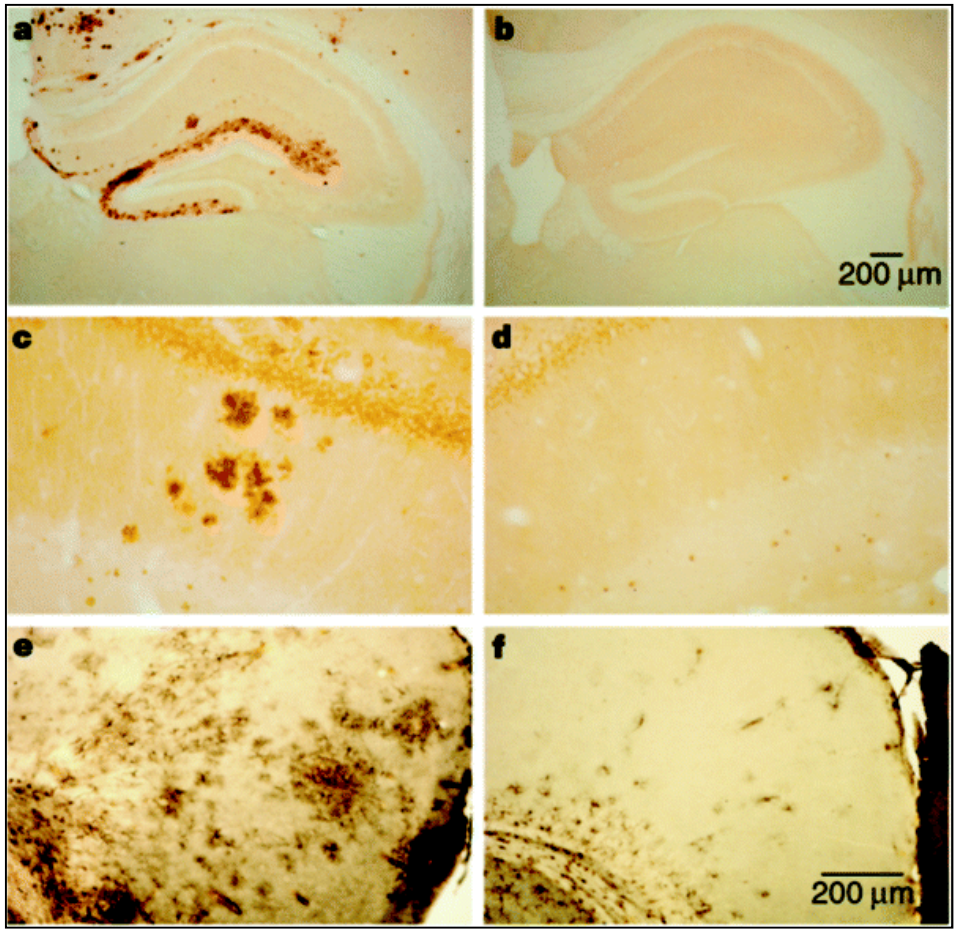
Immunisation with Aß(1-42) produces active antibodies against AD plaques - The initial breakthrough (1999/2000)

Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse

Dale Schenk, Robin Barbour, Whitney Dunn, Grace Gordon, Henry Grajeda, Teresa Guido, Kang Hu, Jiping Huang, Kelly Johnson-Wood, Karen Khan, Dora Kholodenko, Mike Lee, Zhenmei Liao, Ivan Lieberburg, Ruth Motter, Linda Mutter, Ferdie Soriano, George Shopp, Nicki Vasquez, Christopher Vandevert, Shannan Walker, Mark Wogulis, Ted Yednock, Dora Games & Peter Seubert

Elan Pharmaceuticals, 800 Gateway Boulevard, South San Francisco, California 94080, USA

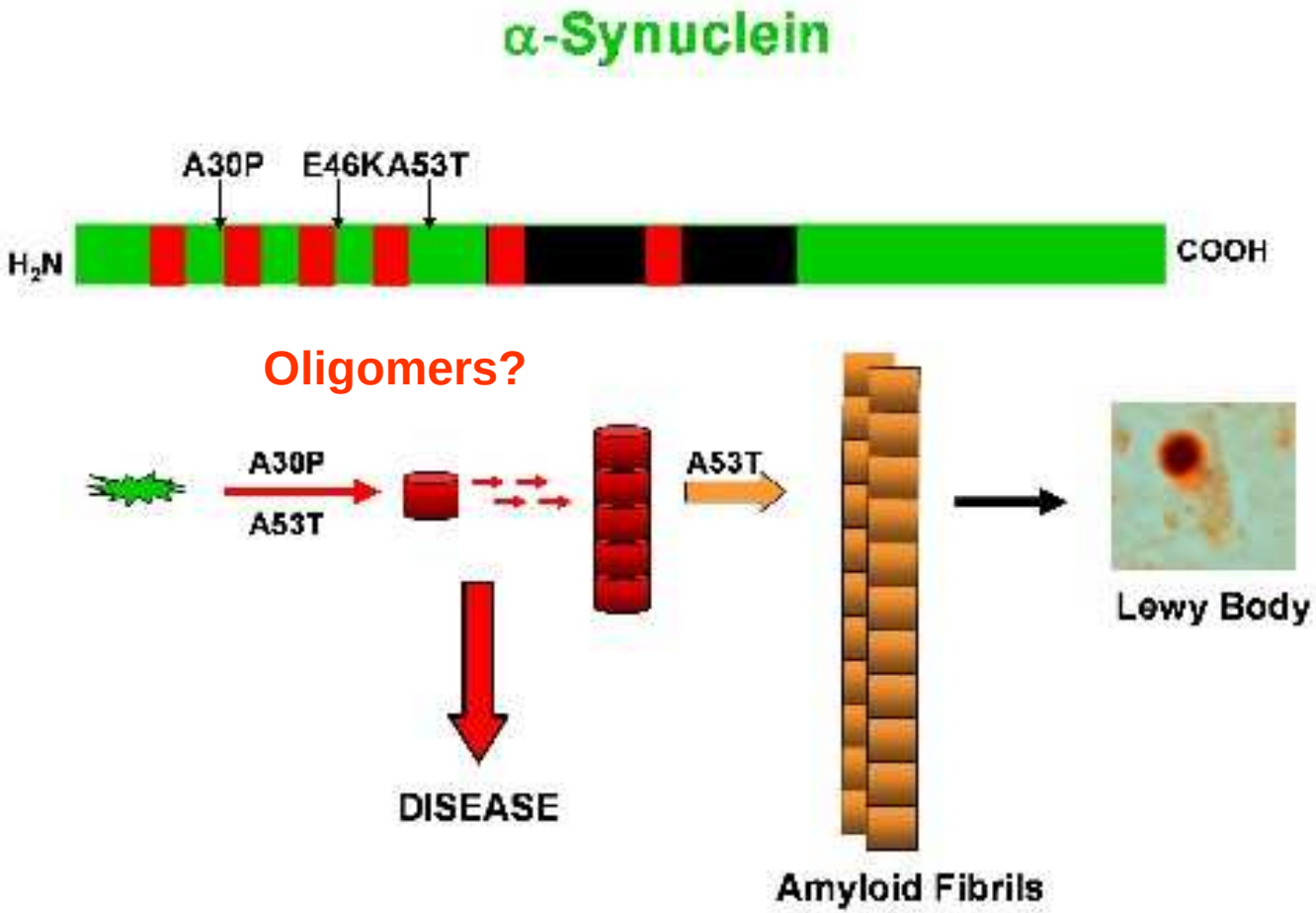


Control Immunisation

Schenk, D. et al. (1999) Nature 400.
Bard, F. et al. (2000) Nat Med. 6.
Weiner, H.L. et al. (2000) Ann Neurol. 48.
Morgan, D. et al. (2000) Nature 408.
DeMattos, R.B. et al. (2001) PNAS USA. 98.

Aggregation and fibril formation of α-Synuclein

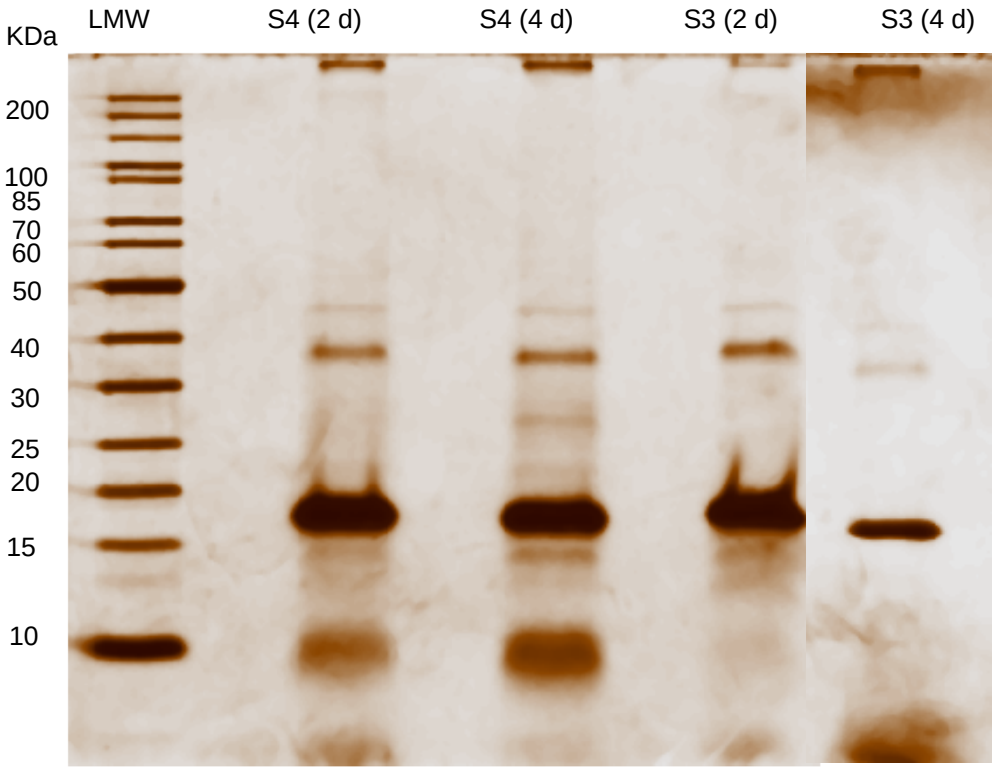
- key protein in Parkinson‘s disease -



Conway, et al Nat Med 1998 *Conway et al PNAS 2000* *Goldberg and Lansbury, Nat Cell Biol 2001*

Alpha- Synuclein shows “oligomers” AND degradation products
Direct mass spectrometry unsuccessful

2 – 6 days



- ü **10 µg aggregates**
- ü **15 % Separation gel**
- ü **5 µl LMW marker**

S3 **α-Syn in ammonium acetate**
S4 **α-Syn in ammonium acid carbonate**

OVERVIEW

I ALPHA-SYNUCLEIN: AUTOPROTEOLYTIC FRAGMENTATION IS KEY INTERMEDIATE OF OLIGOMERIZATION – AGGREGATION
- ION MOBILITY- MS

II SYNTHESIS & AGGREGATION STUDIES OF SYNUCLEIN MUTANTS ONLINE- BIOAFFINITY-MS

Neuronal a-Synuclein – structural details

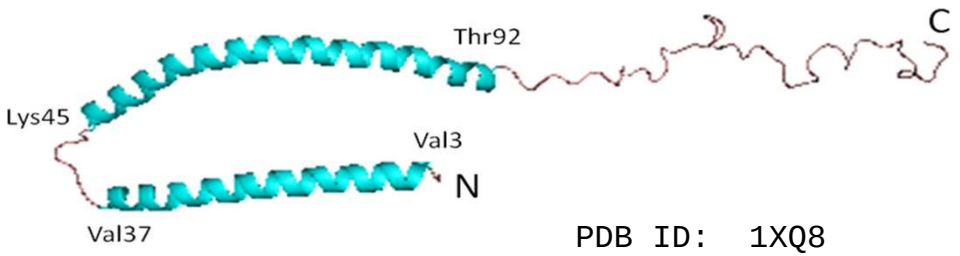
a-Syn wt

```
1   MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV GSKTKEGVVH GVATVAEKTK
61  EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFVKKDQL GKNEEGAPQE GILEDMPVDP
121 DNEAYEMPSE EGYQDYEPEA
```

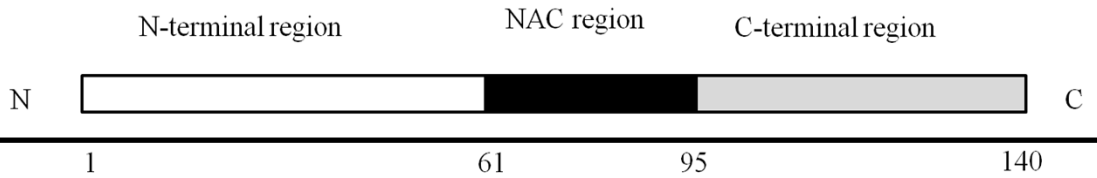
α-Syn mutants

```
                                  A30P                E46K     A53T
1   MDVFMKGLSK AKEGVVAAAE KTKQGVAEAP GKTKEGVLYV GSKTKKGVVH GVTTVAEKTK
61  EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFVKKDQL GKNEEGAPQE GILEDMPVDP
121 DNEAYEMPSE EGYQDYEPEA
```

- member of Synuclein family
- expressed in neurons of CNS
- unstructured and natively unfolded
- Consists of 3 regions: 140 aa (14 kDa)
  - N-terminal region (1-60)
  - Hydrophobic amyloidogenic component (NAC) region (61-94)
  - Negatively charged C-terminal region (96-140)



PDB ID: 1XQ8

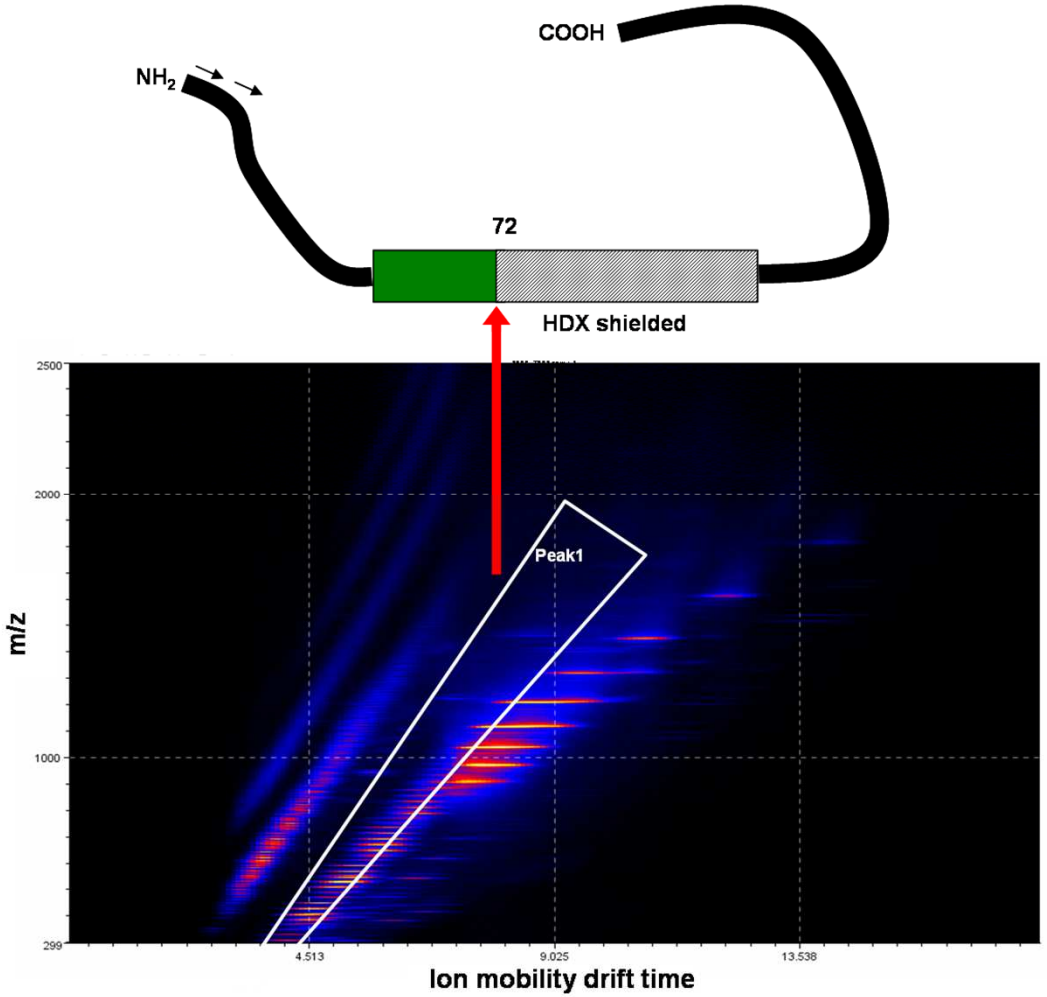


[ChemBioChem 2011]

Autoproteolytic Fragments are Intermediates in the Oligomerization-Aggregation of Parkinson's Disease Protein Alpha-Synuclein as Revealed by Ion Mobility Mass Spectrometry

Camelia Vlad,[a] Kathrin Lindner,[a] Christiaan Karreman,[b] Stefan Schildknecht,[b] Marcel Leist,[b] Nick Tomczyk,[c] John Rontree,[c] James Langridge,[c] Karin Danzer,[d] Thomas Ciossek,[d] Alina Petre,[a,e] Michael L. Gross,[e] Bastian Hengerer,[d] and Michael Przybylski[a]*

[a] *Dr. C. Vlad, MSc K. Lindner, Dr. A. Petre, Prof. Dr. M. Przybylski Department of Chemistry, University of Konstanz 78457 Konstanz (Germany)*
[b] *Dr. C. Karreman, Dr. S. Schildknecht, Prof. Dr. M. Leist Department of Biology, University of Konstanz*
[c] *N. Tomczyk, Dr. J. Rontree, Dr. J. Langridge, Waters Ltd., Micromass Manchester, (UK)*
[d] *Dr. K. Danzer, Dr. T. Ciossek, Prof. Dr. B. Hengerer ZNS Research,Böhringer Ingelheim Pharma,Biberach/Riß (Germany)*
[e] *Dr. A. Petre, Prof. Dr. M. Gross, Department of Chemistry Washington University St. Louis (USA)*



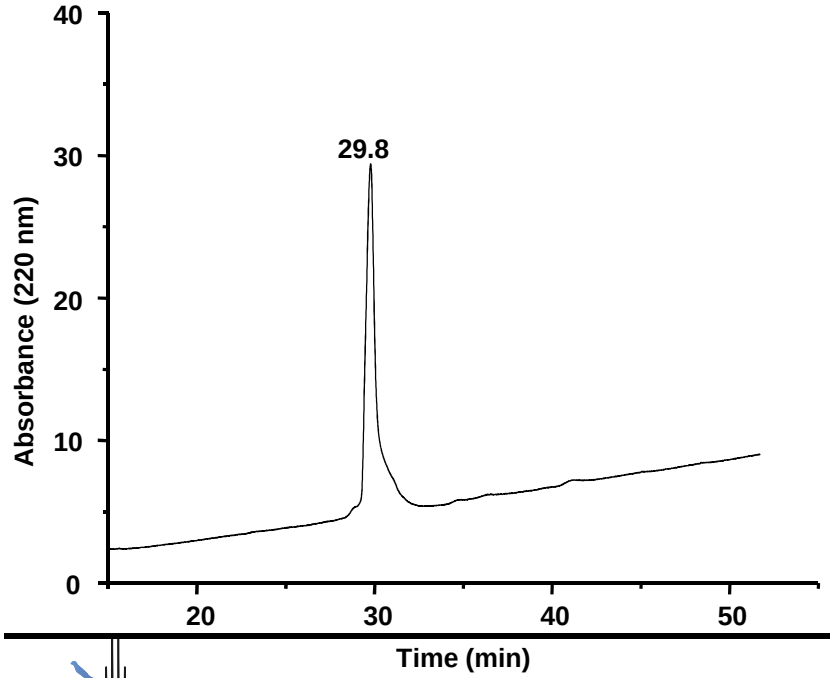
HPLC and ESI- MS of recombinant α-Syn (fresh preparation)

1
MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV
51
GSKTKEGVVH

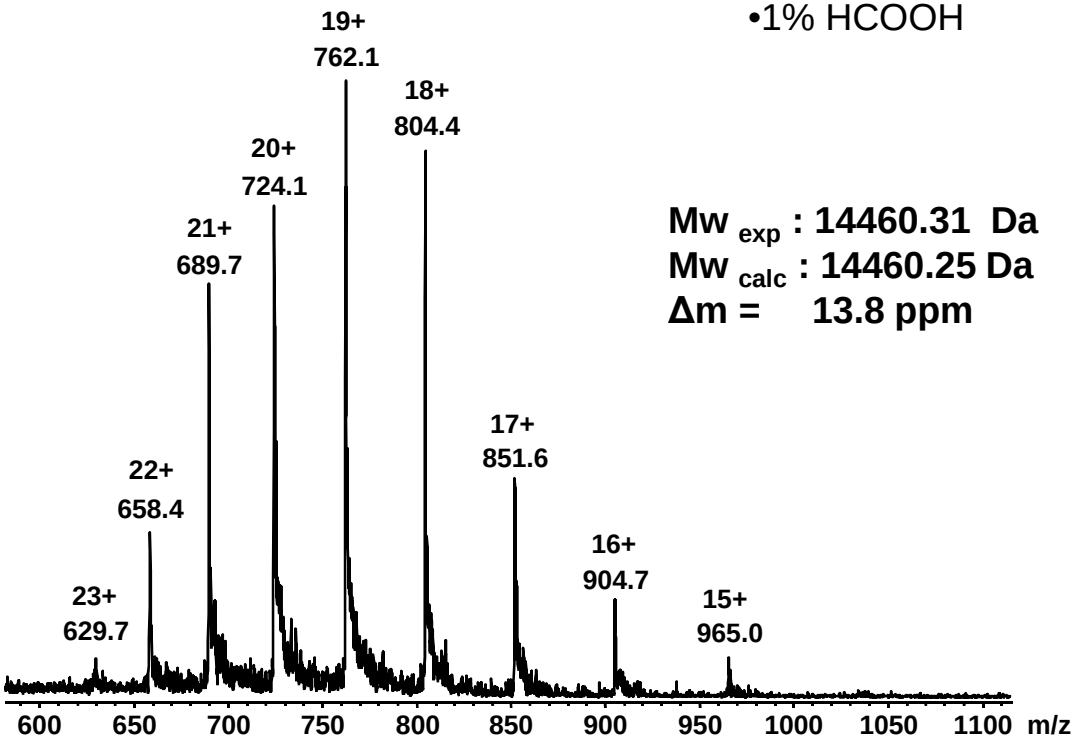
101
GVATVAEKTK EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA
140
ATGFVKKDQL

GKNEEGAPQE GILEDMPVDP DNEAYEMPSE EGYQDYEPEA

- 50 µg (0.1 µg/µL)
- Column C4 Vydac
- λ= 220 nm



- 10 µM
- 1% HCOOH



Mw $_{exp}$: 14460.31 Da
Mw $_{calc}$: 14460.25 Da
Δm = 13.8 ppm

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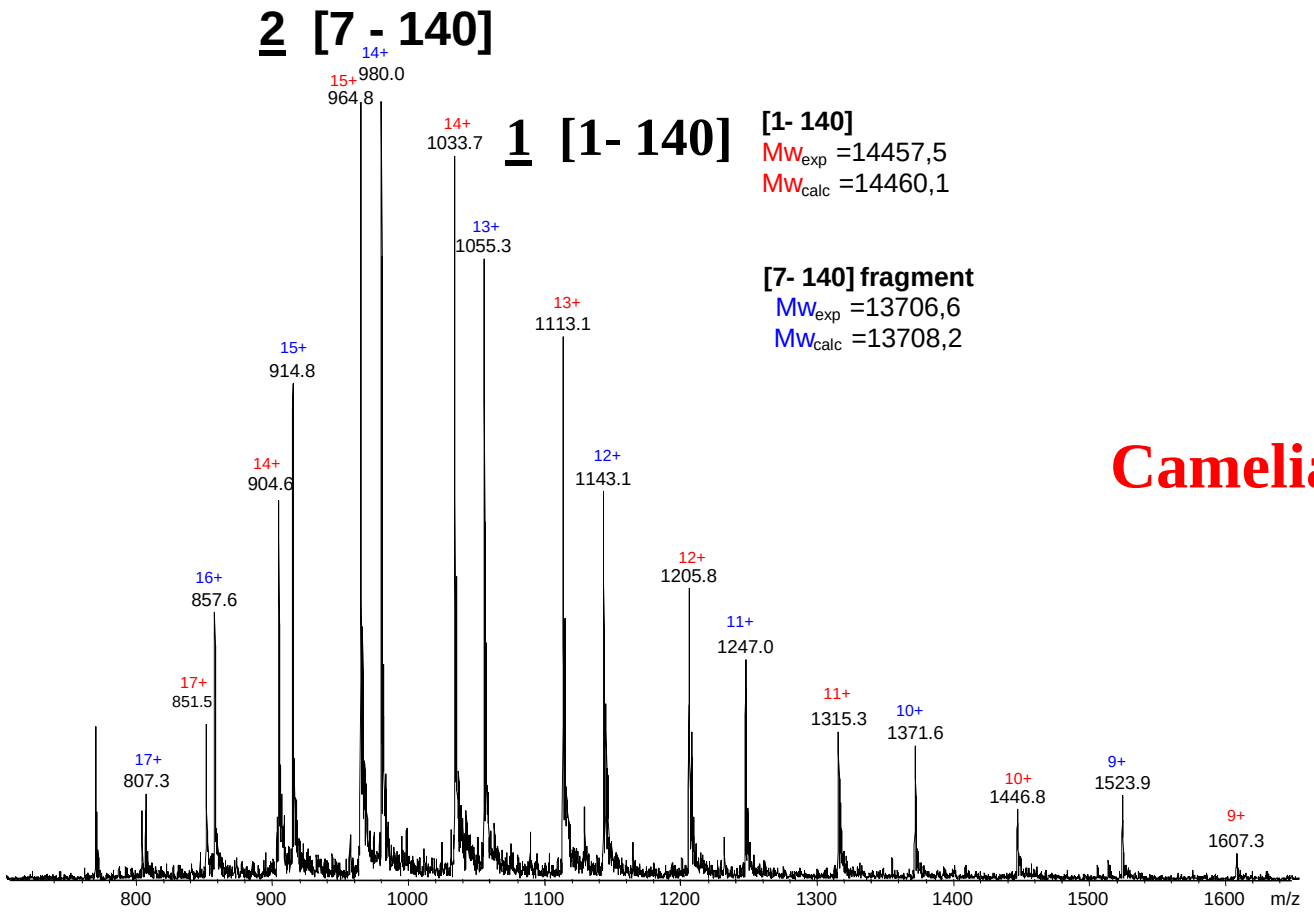
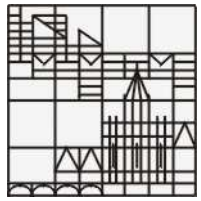
N-Terminal autoproteolytic truncation of alpha-synuclein / 3 hrs

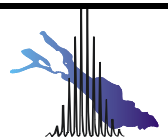


Camelia Vlad

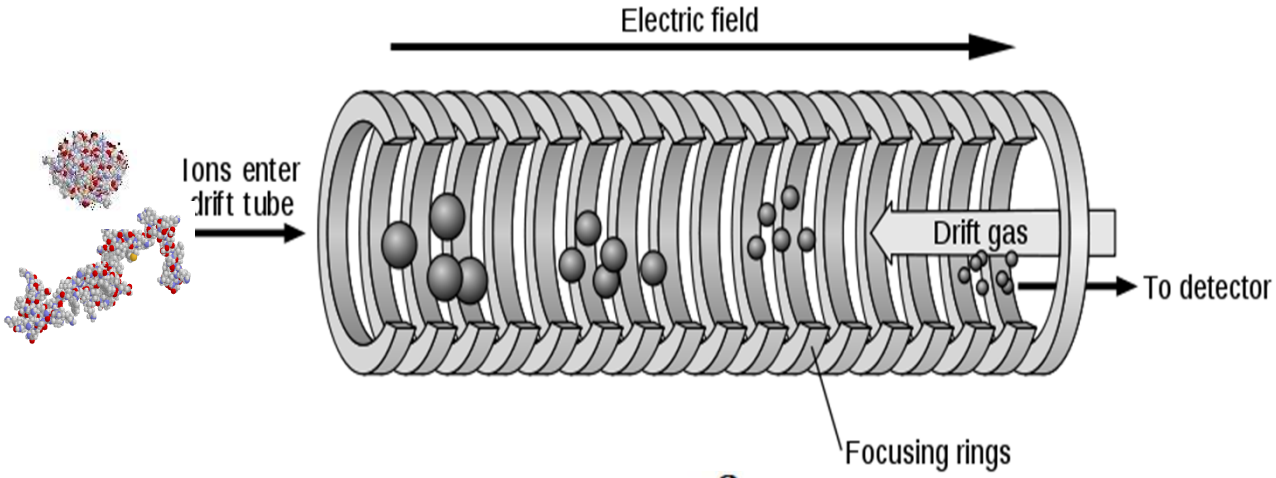
Ion mobility

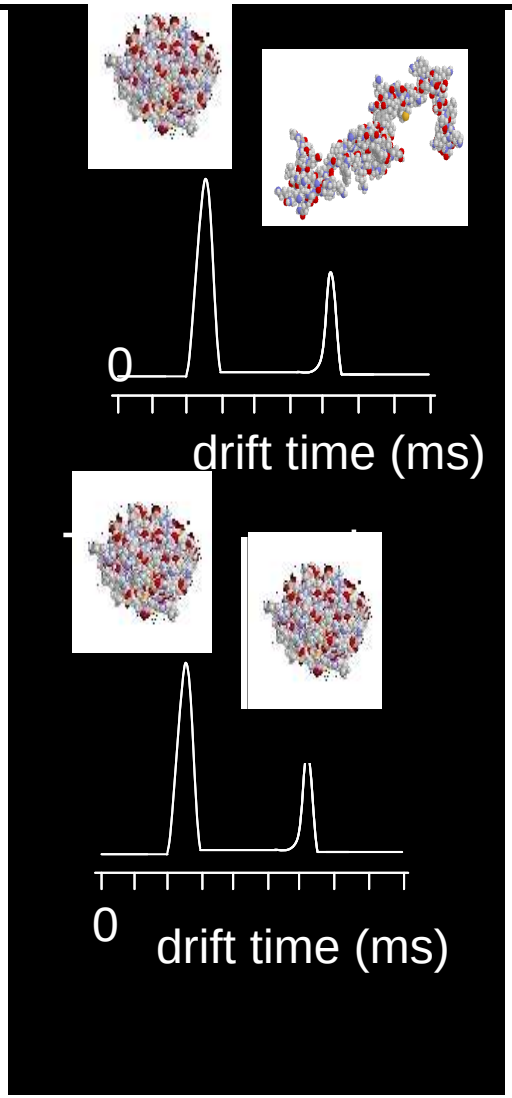


- Separation of ions as they drift through a gas under the influence of an electric field
- Rate of drift is dependant on the ion's mobility through the gas
- Mobility is dependant on factors such as
  - **Size**
  - **Shape**
  - **Charge**
- Ion mobility offers the potential of a **"conformation-specific" separation**
- **- CONCENTRATION- INDEPENDENT**



Ion Mobility Mass Spectrometry – Topology-dependent, Concentration- independent separation





mobility

$$K = \frac{v_D}{E} = \frac{L^2}{t_D V}$$

collision cross section (Å^2)

$$\Omega = \frac{(18\pi)^{1/2}}{16} \frac{ze}{(k_b T)^{1/2}} \left[\frac{1}{m_i} + \frac{1}{m_B}\right]^{1/2} \frac{t_D E}{L} \frac{760}{P} \frac{T}{273.2} \frac{1}{N}$$

Ze: ion's charge

K_b: *Boltzmann's* constant

m_I *and* m_b: mass of the ion and the mass of the buffer gas

t_D *: ion's drift time*

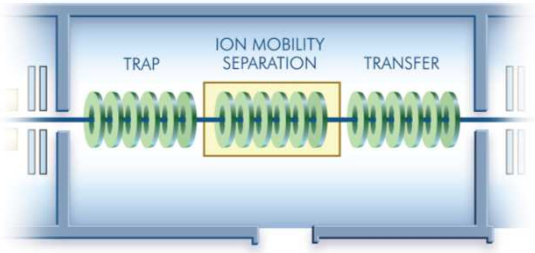
E*: the* electric field strength

L *:*the drift length

Triwave technology for ion mobility separation (Waters Synapt- QTOF)

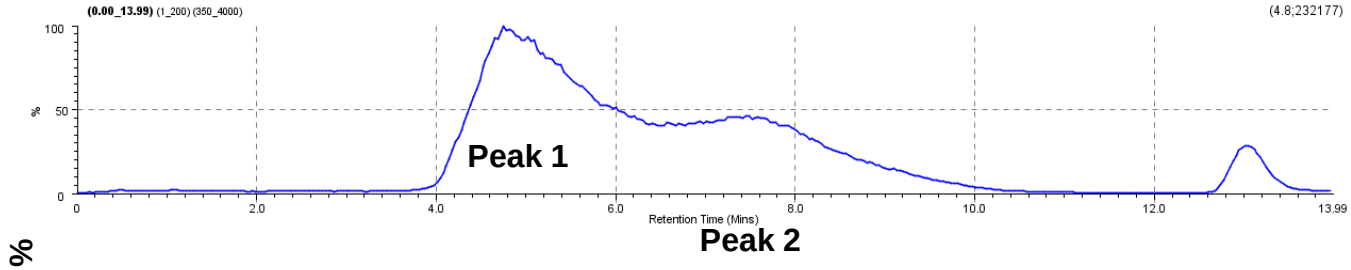
a)

Orthogonal quadrupole

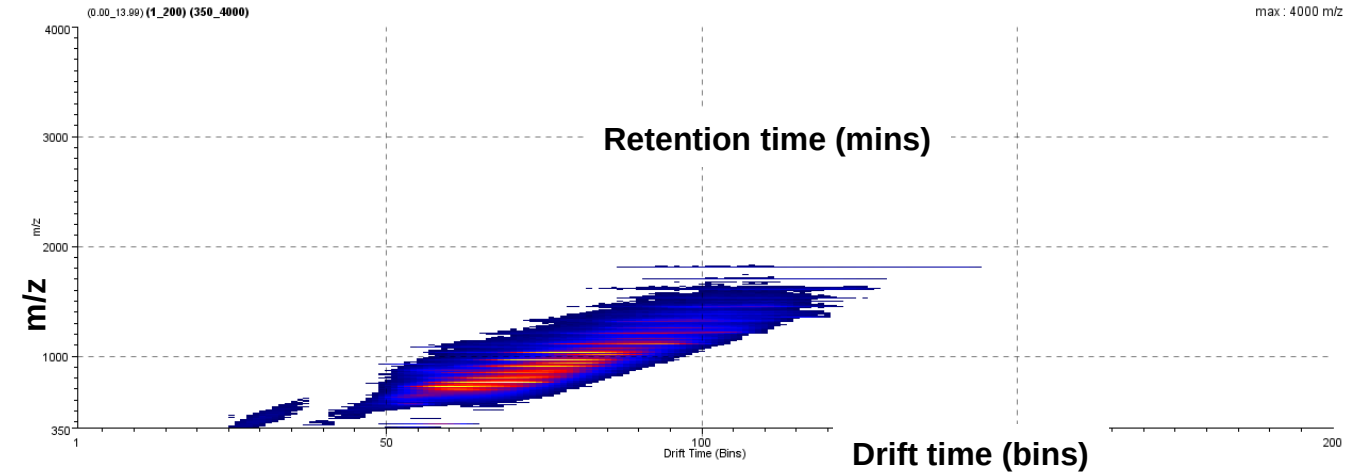


TOF

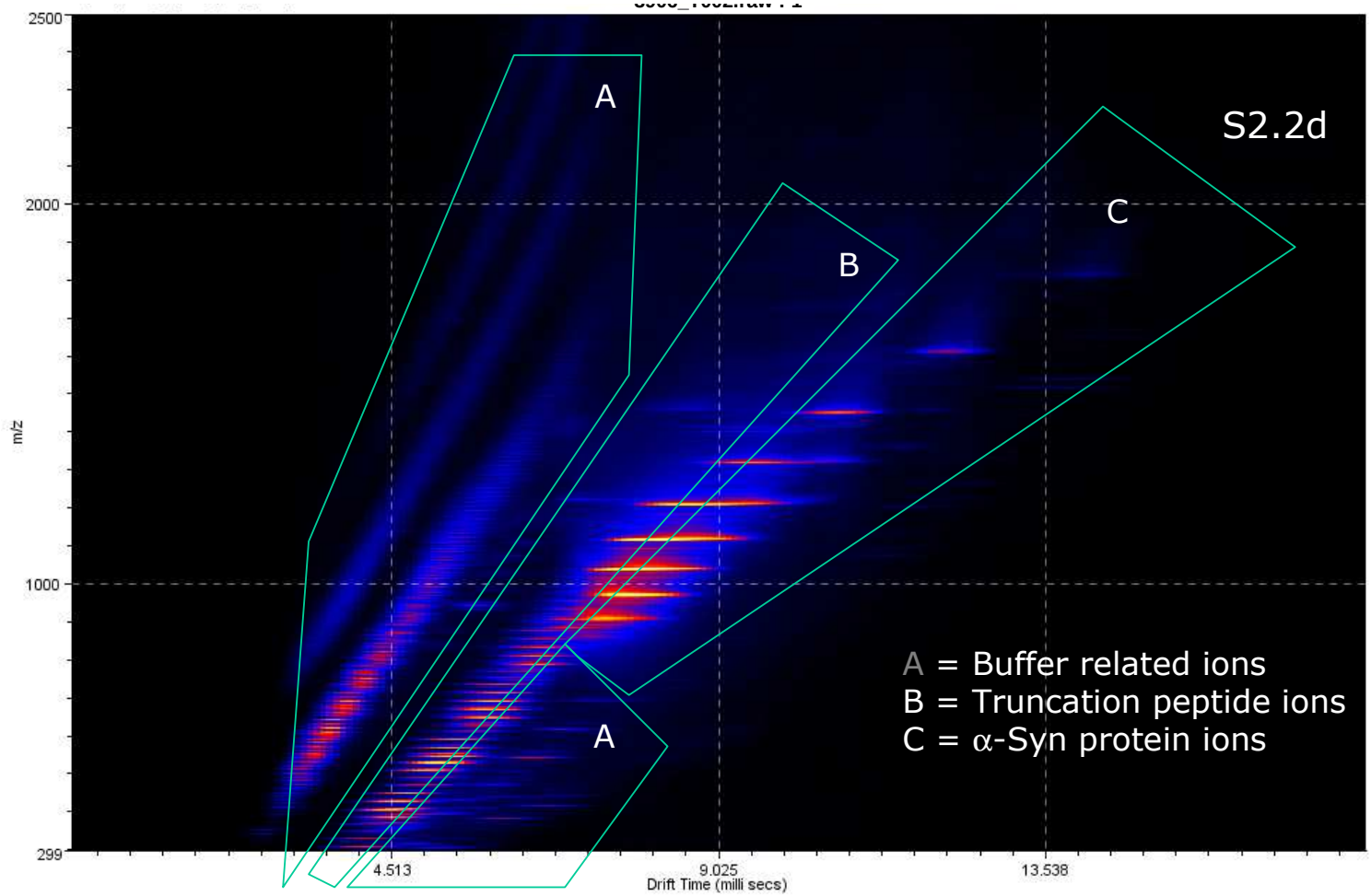
b)



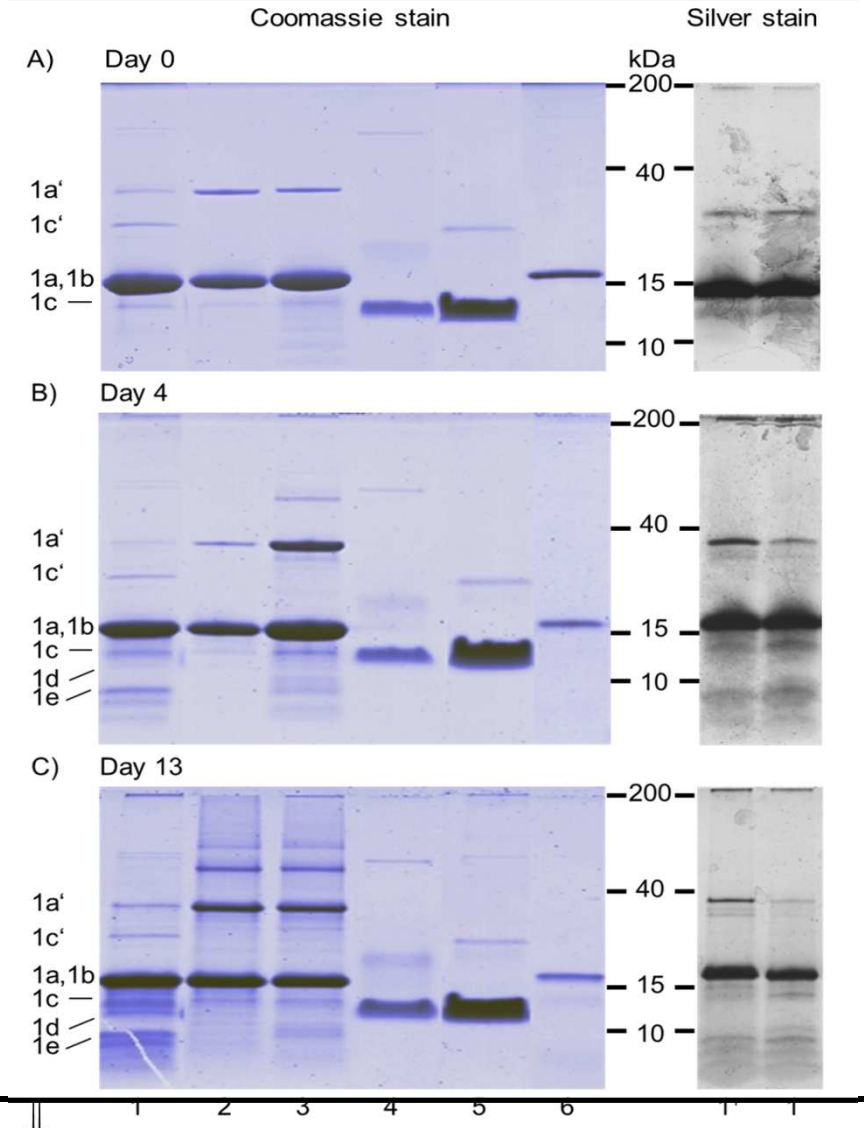
c)



Analysis α-Syn incubated at 37 °C /7 days - Driftscope data view

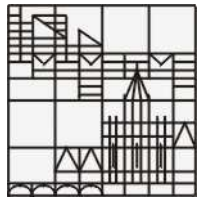


Autoproteolytic fragmentation of α-synuclein - but NOT ß-synuclein



1	wt- a-syn
2	a-syn(70-75)Ala_6
3	a-syn(70-75)Gly_6
4	a-syn(72-140)rc
5	a-syn(72-140)ch
6	ß-syn

ß-Synuclein lacks the central domain (70-72) - VVT and shows no truncation and aggregation



α- Synuclein

10 20 30 40 50

H_2N- MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV GSKTKEGVVH

60 70 80 90 100

GVATVAEKTK EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFVKKDQL

110 120 130 140

GKNEEGAPQE GILEDMPVDP DNEAYEMPSE EGYQDYEPEA - COOH

ß- Synuclein

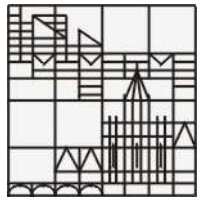
MDVFMKGLSM AKEGVVAAAE KTKQGVTEAA EKTKEGVLYV GSKTREGVVQ

70 80

GVASVAEKTK EQASHLGGAV FSGAGNIAAA TGLVKREEFP TDLKPEEVAQ

EAAEEPLIEP LMEPEGESYE DPPQEEYQEY EPEA

Selective mutation of key sequence in α-syn



α-syn wt

^{1}MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKT

KEGVVHGVATVAEKTKEQVTNVGGA^{70}**VVT**^{72}GVTAVAQKTVEGAG

SIAAATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEE

GYQDYEPEA^{140}

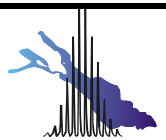
α-syn ^{70}NAN^{72}

^{1}MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKT

KEGVVHGVATVAEKTKEQVTNVGGA^{70}**NAN**^{72}GVTAVAQKTVEGA

GSIAAATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSE

EGYQDYEPEA^{140}

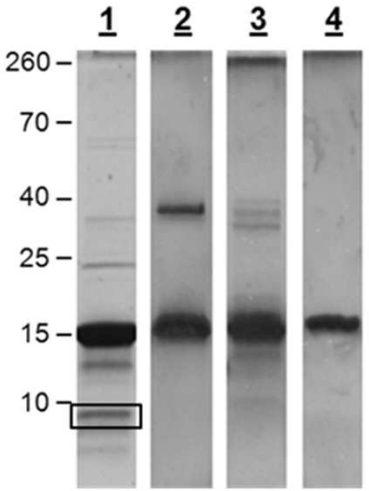


Fragmentation & Aggregation of physiological and pathological Synucleins: The beta-breaking triplett VVT(70-72)

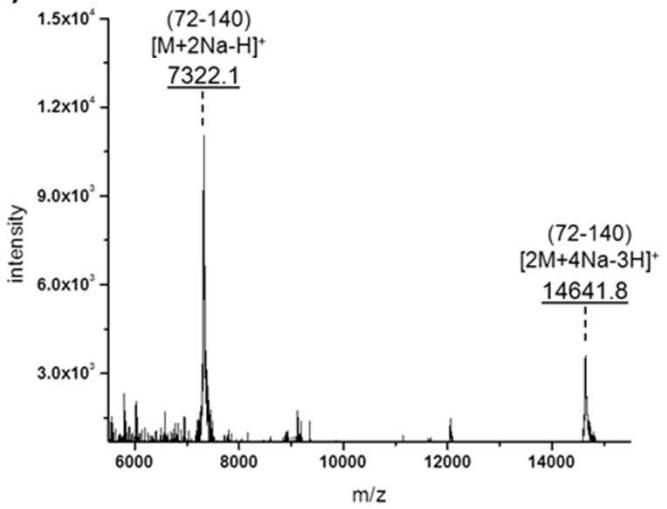
A)

1 αSyn wt	^{1}M…K^{61}EQVTNVGGA^{70}**VVT**^{73}GVTAVAQKTVEGAGSIA^{90}A…^{140}A
2 αSyn NAN	^{1}M…K^{61}EQVTNVGGA^{70}**NAN**^{73}GVTAVAQKTVEGAGSIA^{90}A…^{140}A
3 αSyn VFS	^{1}M…K^{61}EQVTNVGGA^{70}**VFS**^{73}GVTAVAQKTVEGAGSIA^{90}A…^{140}A
4 βSyn	^{1}M…K^{61}EQASHLGGA^{70}**VFS** - - - - - - - - - - -^{73}GAGNIA^{79}A…^{134}A

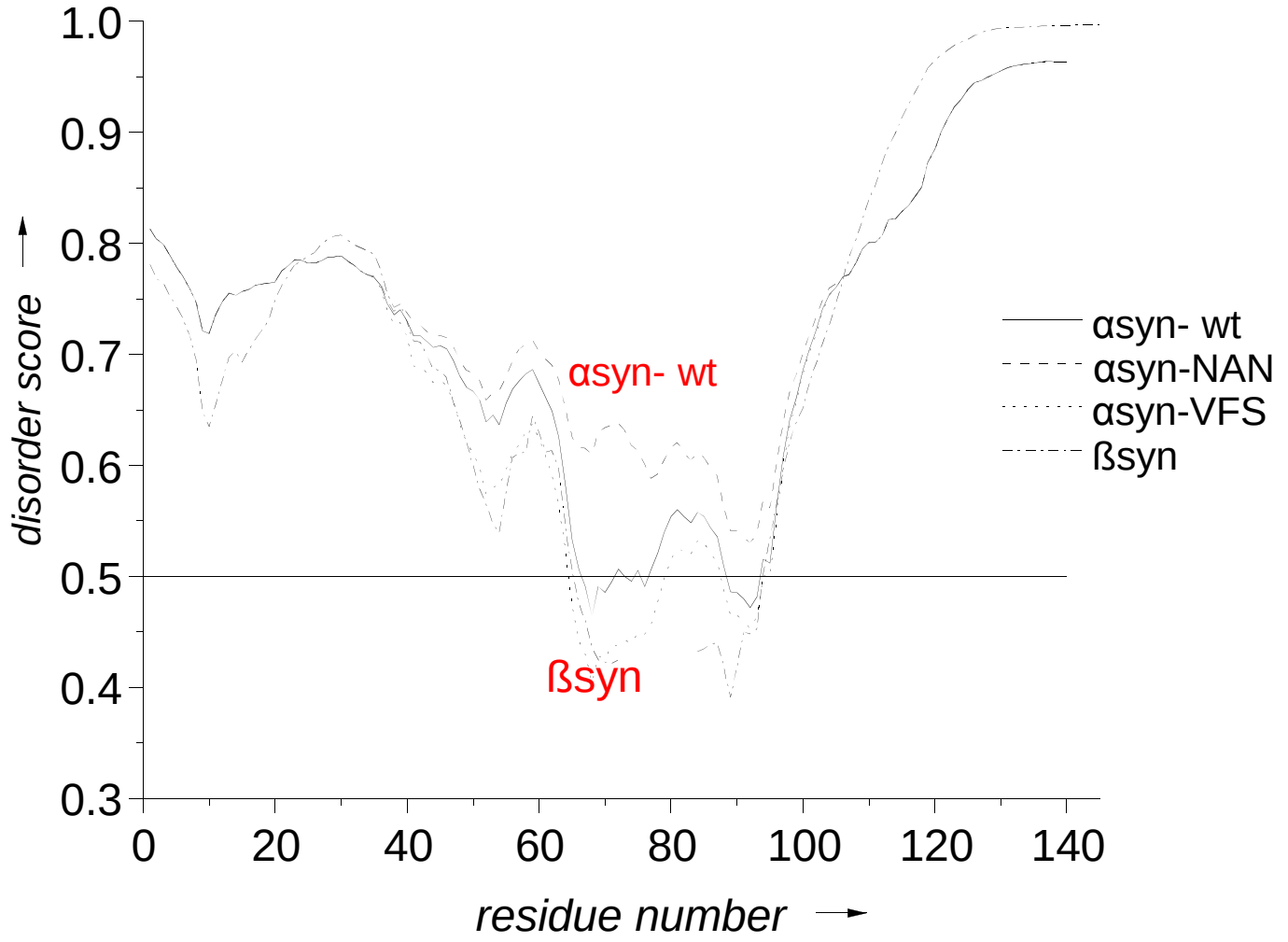
B)



C)



Alpha- and ß-synucleins differ by the highly disordered (70-72)VVT domain
Intrinsical disorder degree



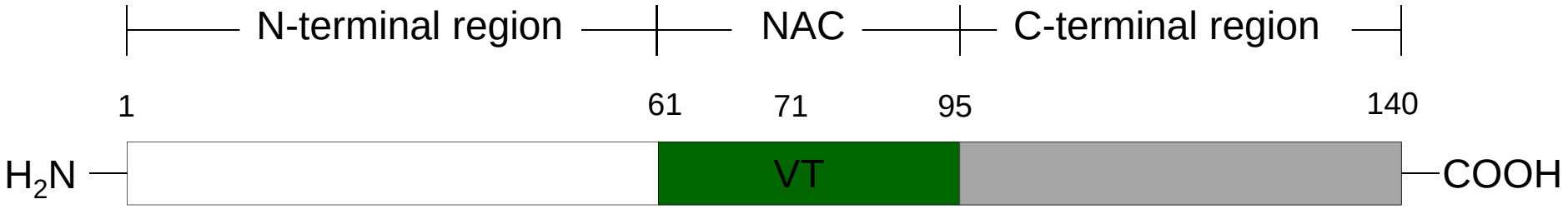
Disorder prediction of human aSyn wt, aSyn(70-72)-mutants and ßSyn by PONDR-VSL2B algorithm. The horizontal line at 0.5 represents a threshold for disordered/ordered residues. Residues above 0.5 are predicted disordered, while residues below 0.5 are predicted ordered. The discontinuous region of ßSyn (dashed line) is due to the lack of 11 amino acids (73-83).

•**Synthesis & aggregation studies of α-synuclein fragments**

Camelia Vlad

Kathrin Lindner

Synthesis of α-Synuclein by: - chemical Synthesis (SPPS)
- recombinant gene expression

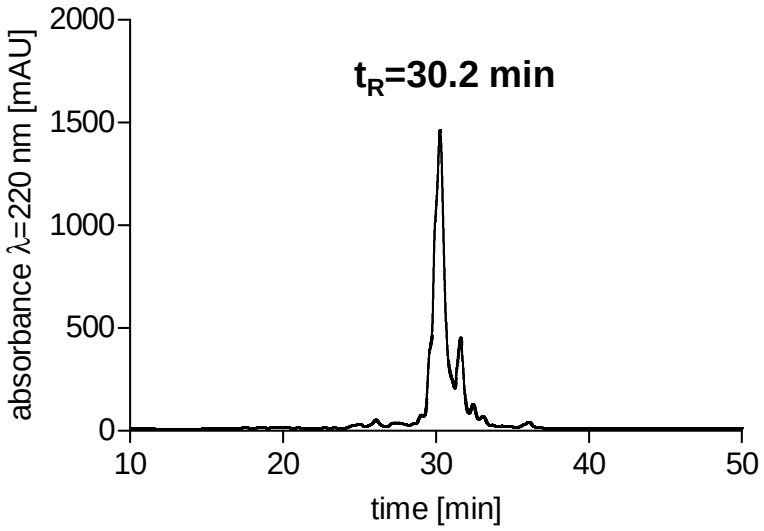


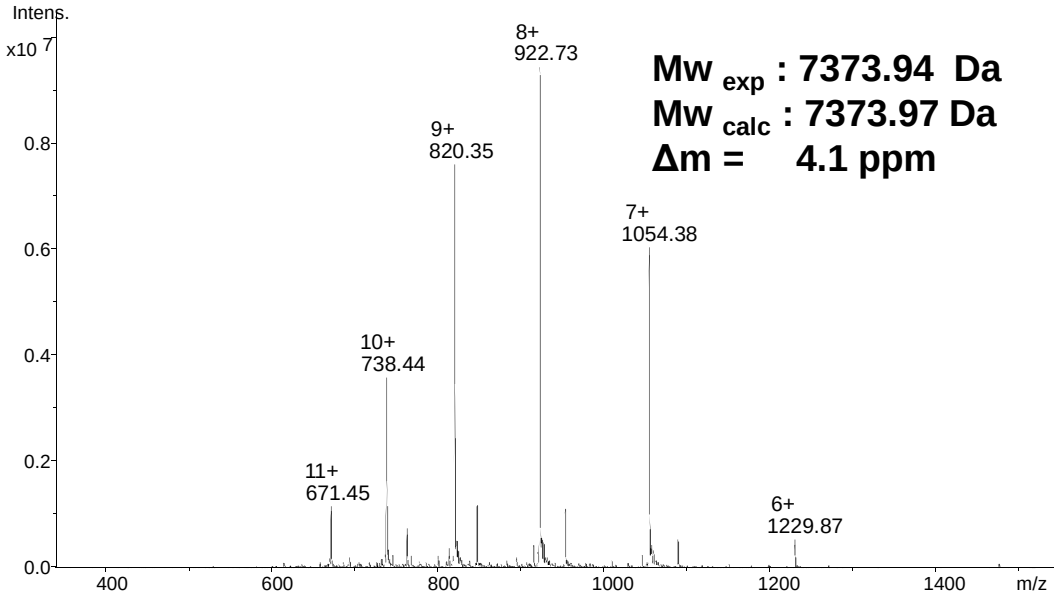
Analytical RP-HPLC and ESI- MS of recombinant α-Syn (71-140)

- *E.Coli* BL21(DE3)[pLys] strain using T7 RNA polymerase system
- centrifugation and resuspend in PBS
- heated to 100°C for 2 min; centrifugation at 4300 rcf for 15 min and resuspend in PBS

•Column C4 Vydac
•λ= 220 nm
•Gradient: 0-5 min 20 %B, 5-50 min 1%/min

•10 µM
•1% HCOOH





Fibrillization kinetics - ThT fluorescence assay: Substantially enhanced aggregation rate of αSyn(71-140)

96well Blackplates (Perkin Elmer)

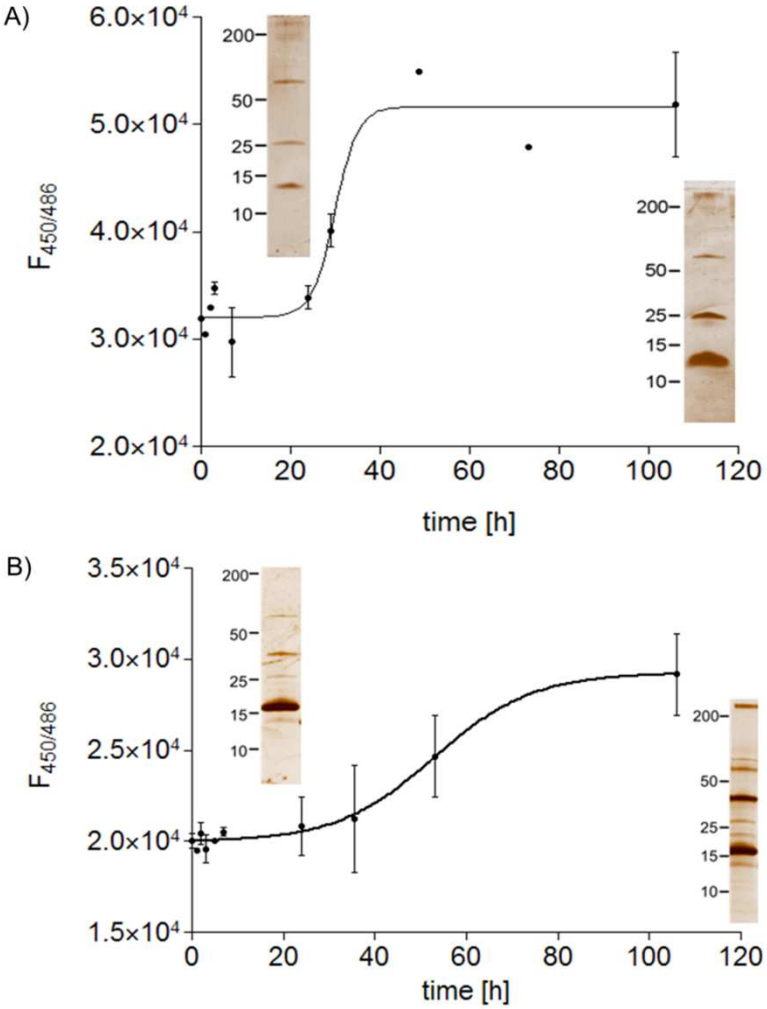
25 µM ThT
7 µM α-Syn

100 µl volume

Fluorescence Parameter:
ex 486 nm
em 450 nm
measurement time 1s

α-Syn (72-140) **4**

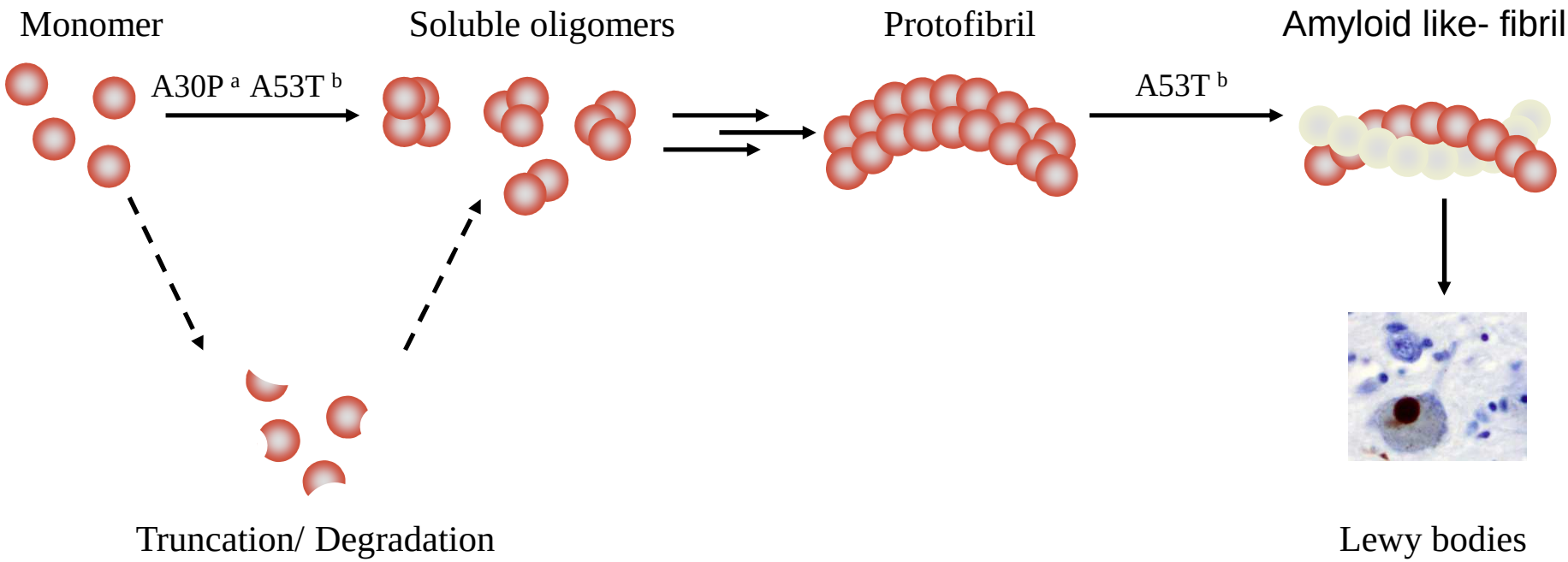
full-length α-Syn **1**



K. Lindner et al. (2011)

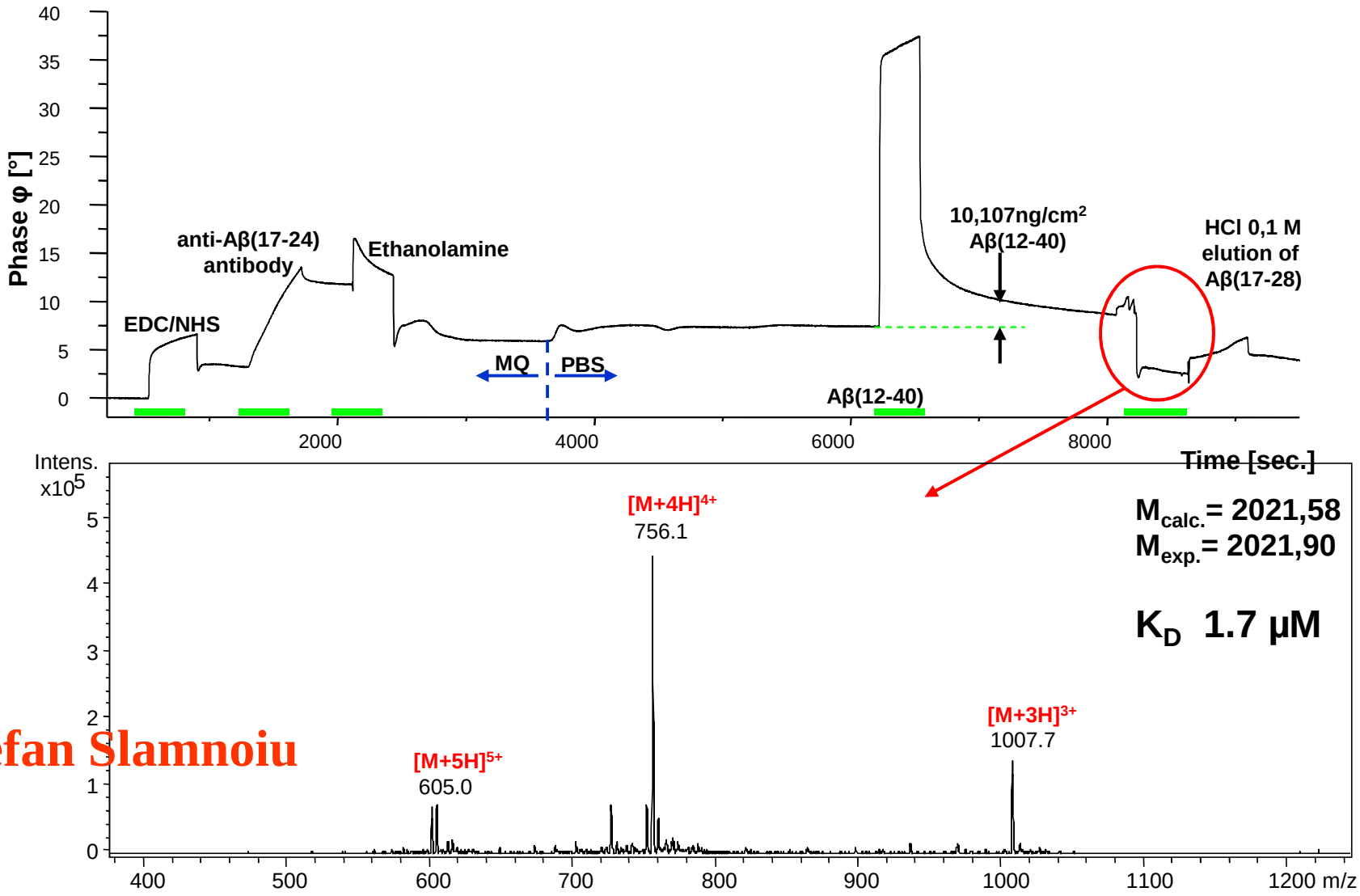
Analytical Chemistry & Biopolymer Structure Analysis
University of Konstanz

αSyn fragmentation - aggregation – Key Mechanism in Vivo?



A30P [a] - promotes the formation of oligomers
A53T [b] - promotes the formation of fibrils

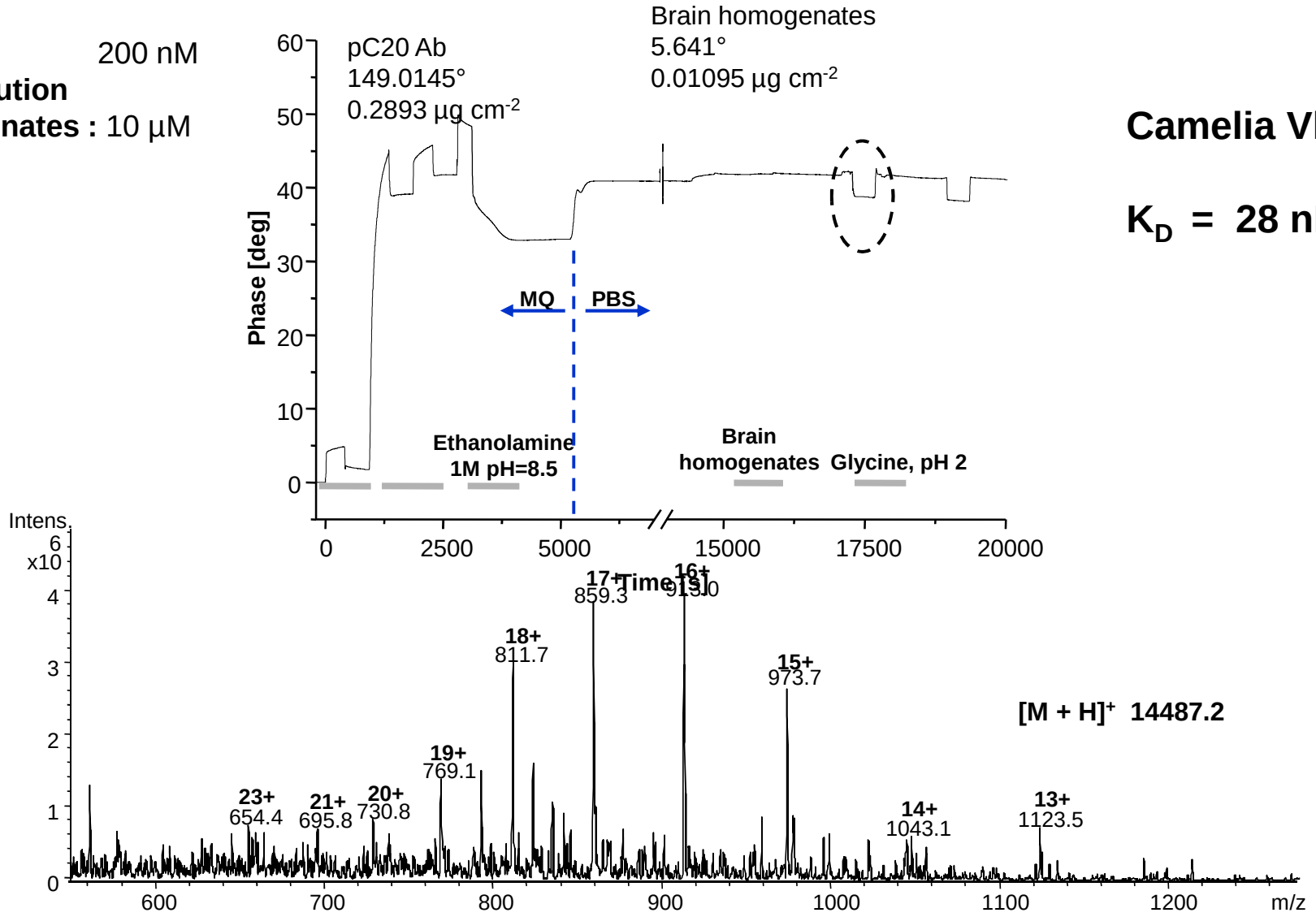
Epitope specificity of anti-Aβ(1-16) antibody by online SAW – ESI MS



Stefan Slamnoiu

Online Affinity-MS of alpha-Syn-m130 mutant
Direct analysis from brain homogenate

pC20: 200 nM
Brain elution homogenates : 10 µM

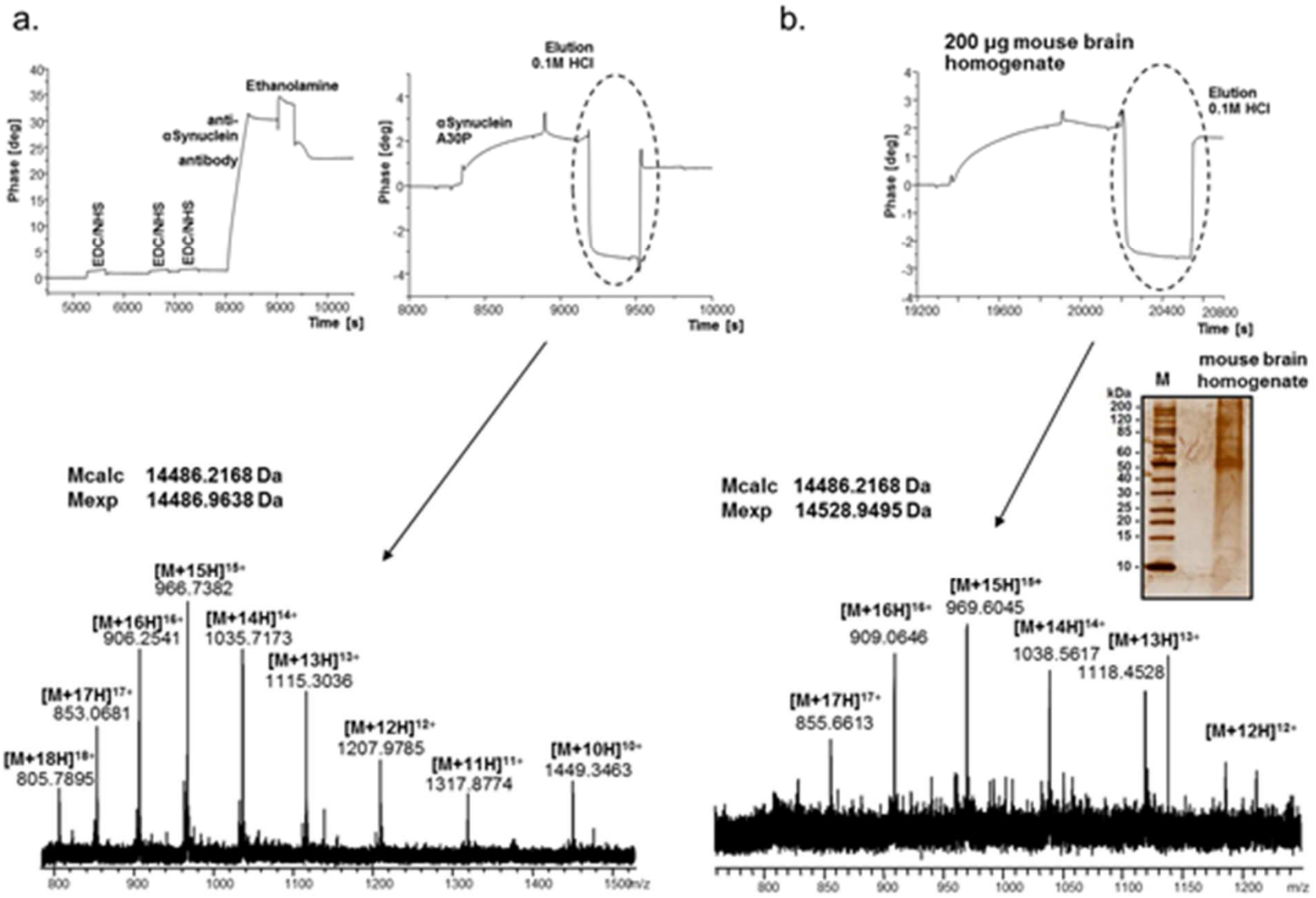


Camelia Vlad

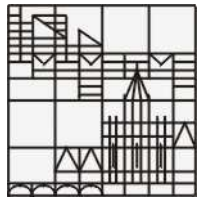
K_D = 28 nM

Online SAW-affinity-MS of wt-aSyn in vitro (a) and from mouse brain homogenate (b)

Figure 3

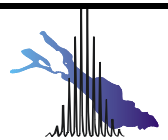


Perspectives for affinity- mass spectrometry / Ion Mobility- MS

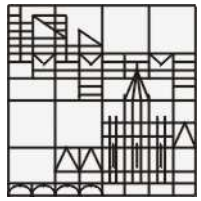


Online Affinity- Mass spectrometry
« Affinity-like » separation by Ion Mobility- MS:

- Identification of antigen epitopes - vaccine lead structures
Biomarker identification
Ligand- binder recognition & interaction
Conformational/topography characterisation
Reactive intermediates in misfolding & aggregation



THANKS TO THE MAJOR PLAYERS...
… Coworkers, Collaborators, €€€...



Coworkers

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David Clemmer, Indiana University
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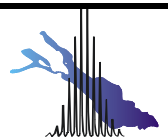
€€€

DFG	Biopolymer-MS & ChemBio Grad School
EU	Antibodies to Human Proteome; RUBICON
Boehringer – Ingelheim,	Parkinson/ Synuclein
Univ. Konstanz	Research Center Proteostasis
BMWI	Affinity-MS