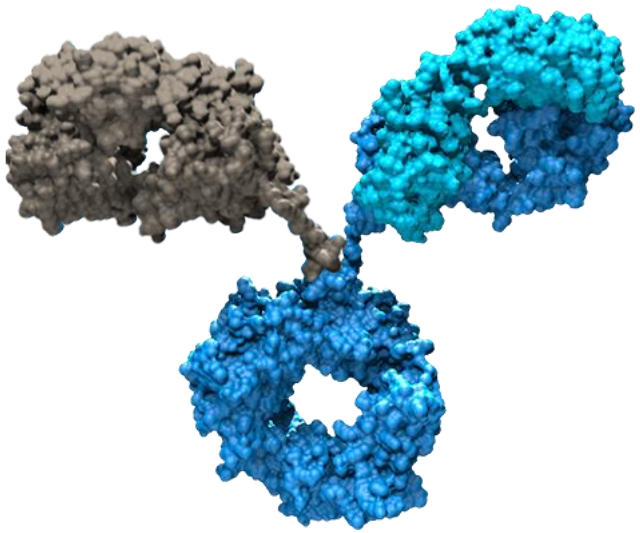
Leiden University
Medical Center

Characterization of new antibody-derived therapeutics at the intact and middle-up level of analysis using sheathless CE-MS



Elena Dominguez-Vega

Center for proteomics and metabolomics

AT EUROPE

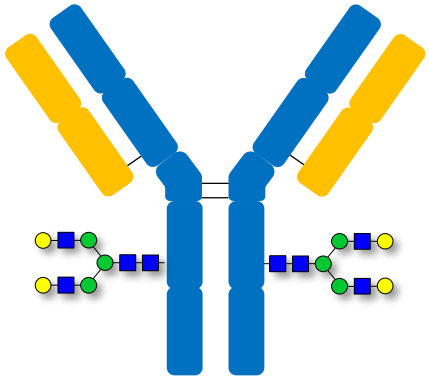
DUBLIN, 13-MARCH-2019

Monoclonal antibodies

- Most selling pharmaceuticals: eg. Adalimumab, Infliximab, Rituximab, Trastuzumab

Microheterogeneity

- N-glycan variability
- Post-translational modifications (PTMs)
- incomplete disulfide bond formation
- …



Macroheterogeneity

- Aggregation
- Free chains (*e.g.* free LC, free HC)
- Partial cleavage
- …

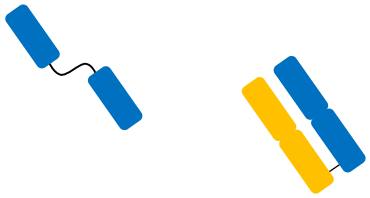
Analytical characterization

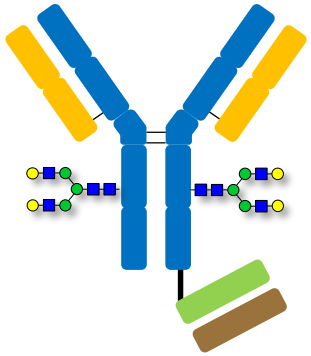
RPLC | **HILIC** | **CIEF** | **IEX** | **CE-SDS** | **SEC**

MS

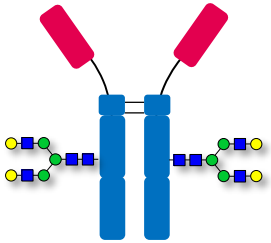
New antibody formats

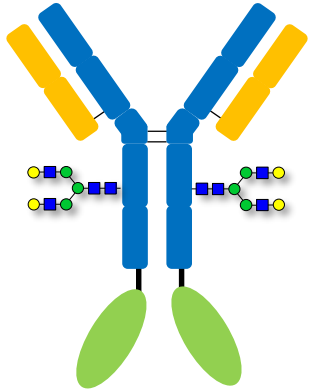
Antibody fragments



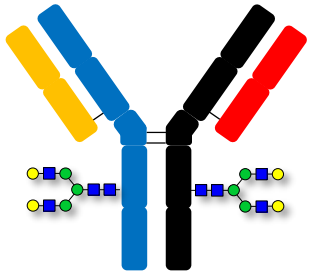


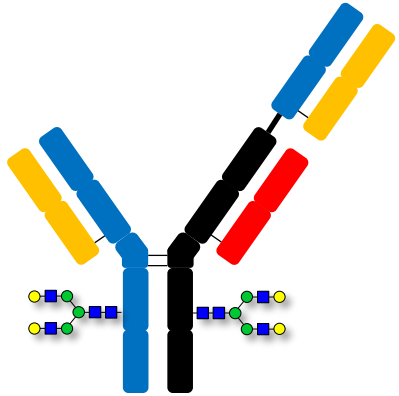
Fusion proteins

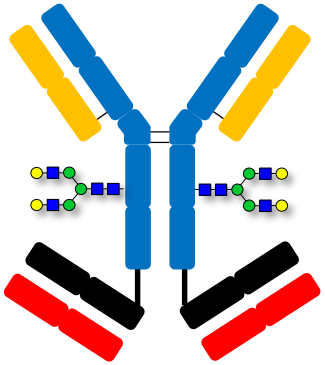




Bispecific antibodies

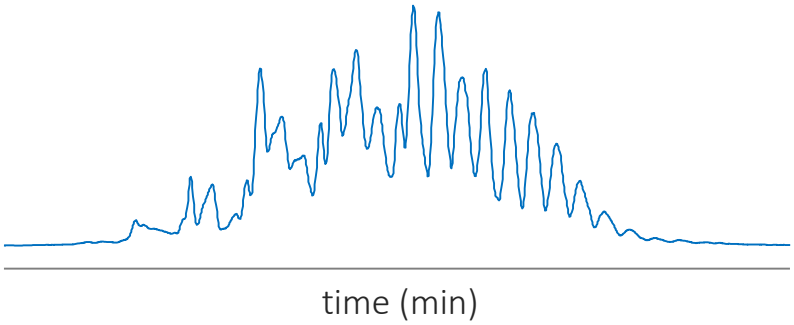






CE for the separation of intact proteins

$$\mu_{electro} = \frac{z}{6\pi\eta r} \cong C\frac{z}{M^{\alpha}}$$



- high separation efficiency
- small diffusion coefficient is advantage
- reveal subtle protein differences/changes

Intact protein analysis

Assessment of protein heterogeneity

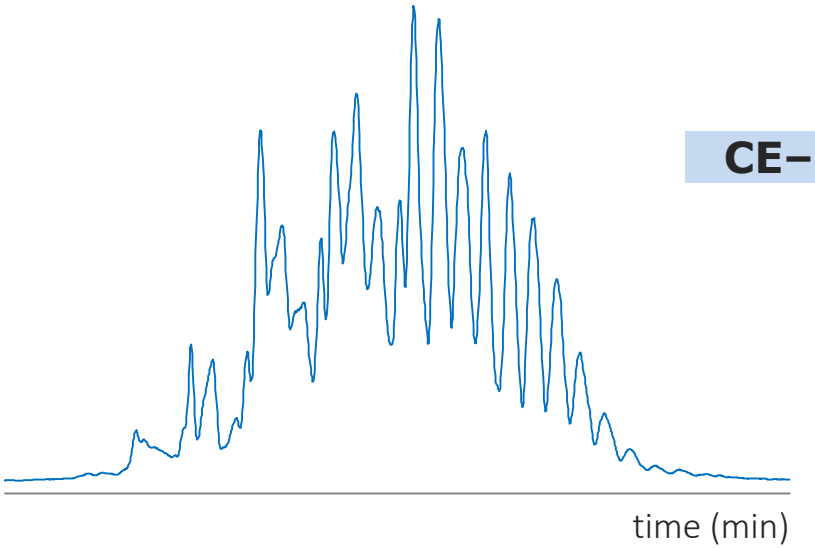
- no stationary phase; free solution, no interaction
- denaturing and native separation

Versatility

CE provides good opportunities to characterize new antibody formats

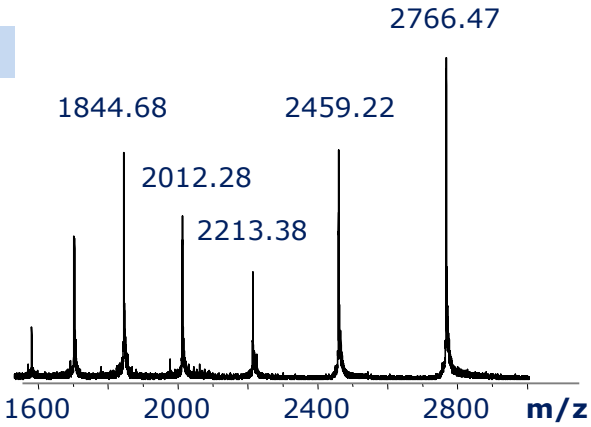
CE-MS of intact proteins

capillary electrophoresis



CE–ESI-MS

mass spectrometry



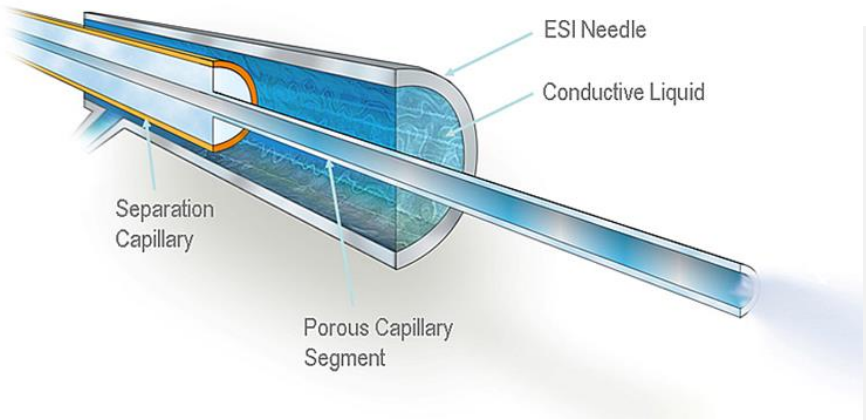
efficient intact protein separation

- high separation efficiency
- small diffusion coefficient is advantage
- reveal subtle protein differences/change
- allows native conditions
- mass loadability of CE is limited

protein assignment

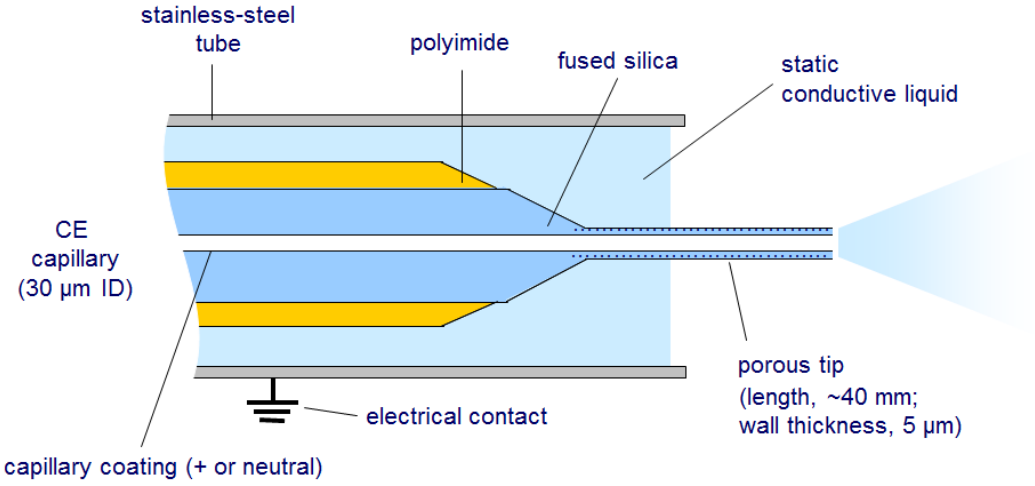
- accurate mass information on intact proteins
- difficult ionization of intact proteins
- ESI of intact protein yields number of multiply charged ions

Sheathless CE-MS (CESI-MS)



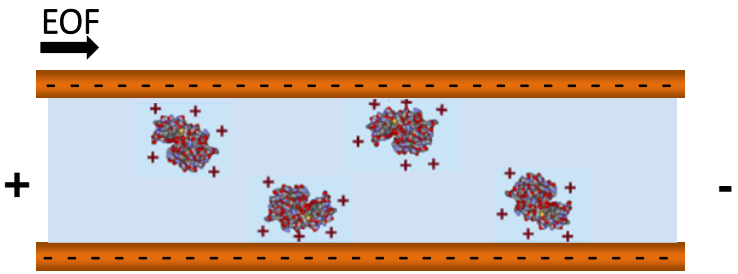
- improves ionization efficiency by nanospray
- no additional liquid:
  - avoids dilution (increased sensitivity)
  - decreases noise
  - allows native MS (conformation studies)

SCIEX



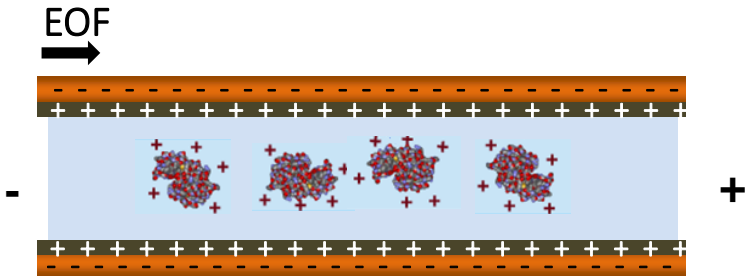
Avoiding protein adsorption in CE(-MS)

pH BGE < pI protein



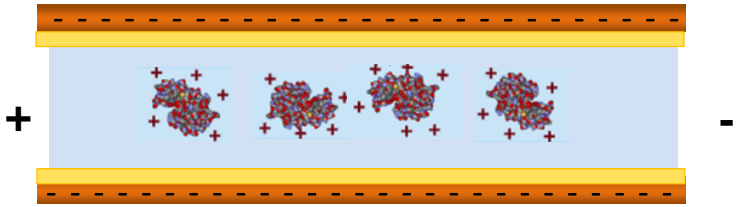
Capillary coatings for CESI-MS

Positive coatings: PEI



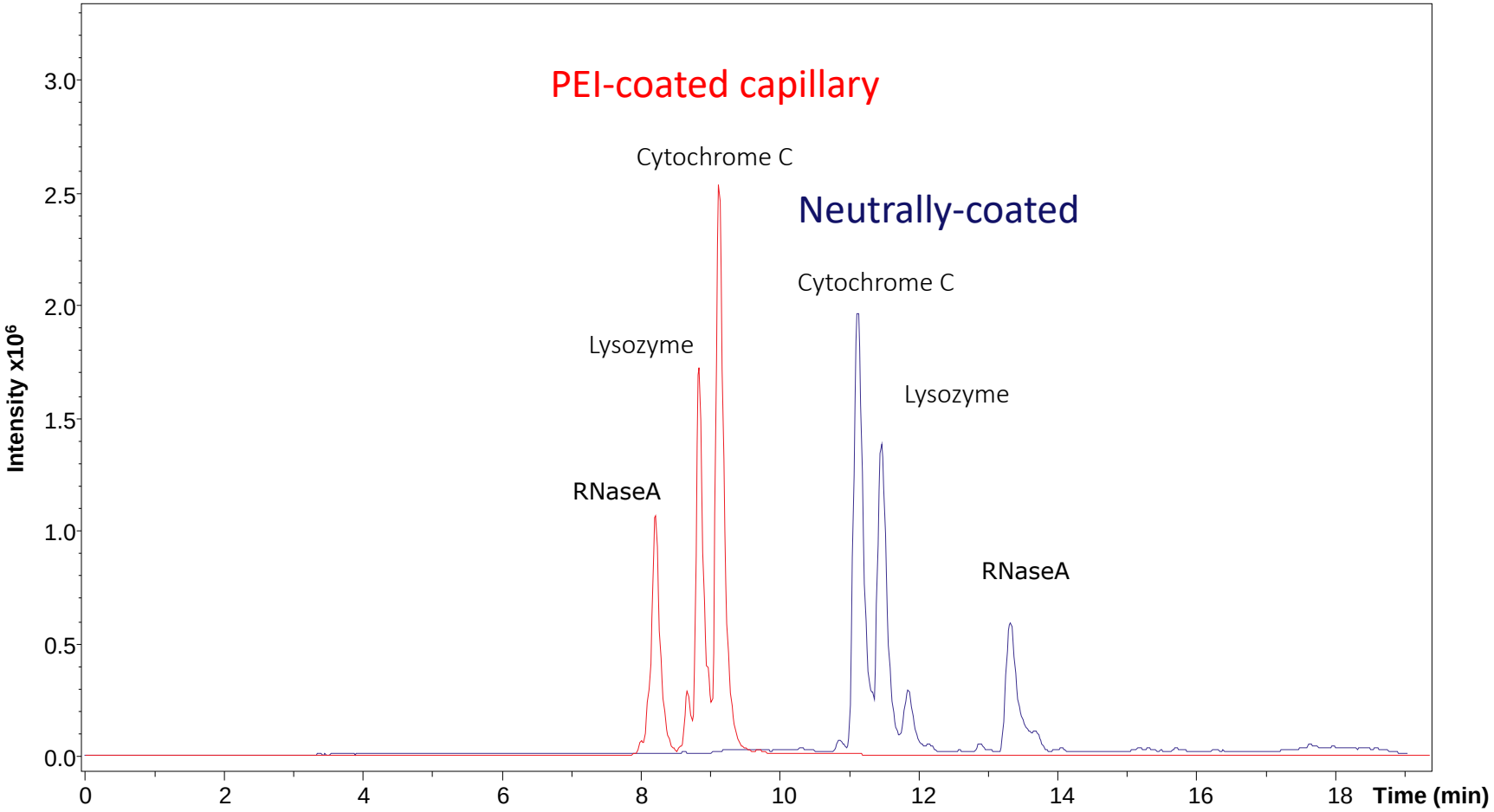
Neutral coatings

No EOF

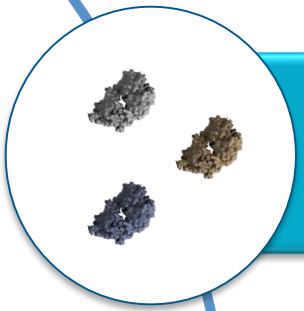


CESI-MS systems for intact protein analysis

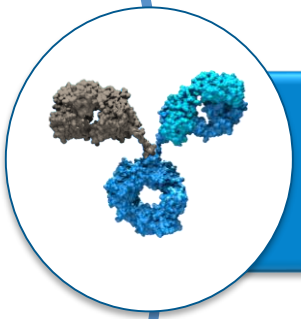
- Protein test mixture (RNaseA, Lysozyme and Cytochrome C, 0.2 μg/μL each)
- BGE 50 mM ammonium acetate pH 3.0



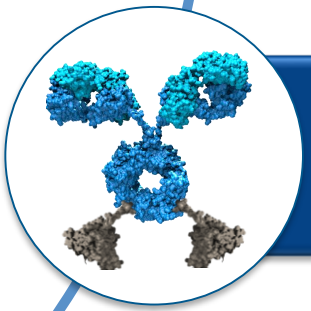
Outline



NANOBODIES



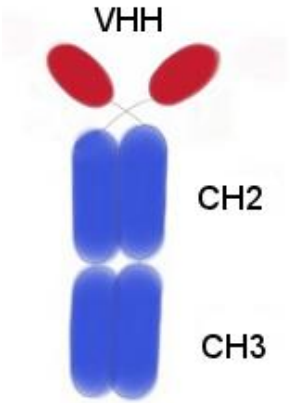
BISPECIFIC ANTIBODIES



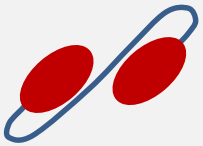
FUSION PROTEINS

Nanobodies

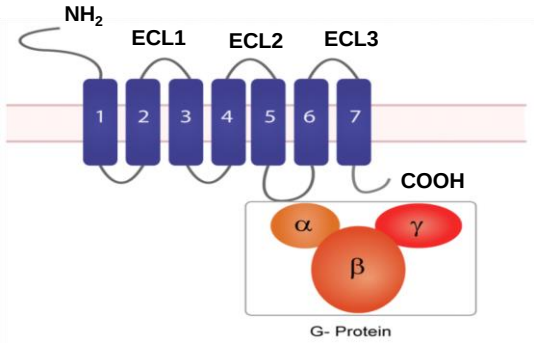
Cameloid antibodies



~100 kDa

monovalent nanobody	bivalent nanobody
	
~15 kDa	~30 kDa

35GS linker
-$(GGGGS)_7$-



G-protein coupled receptors

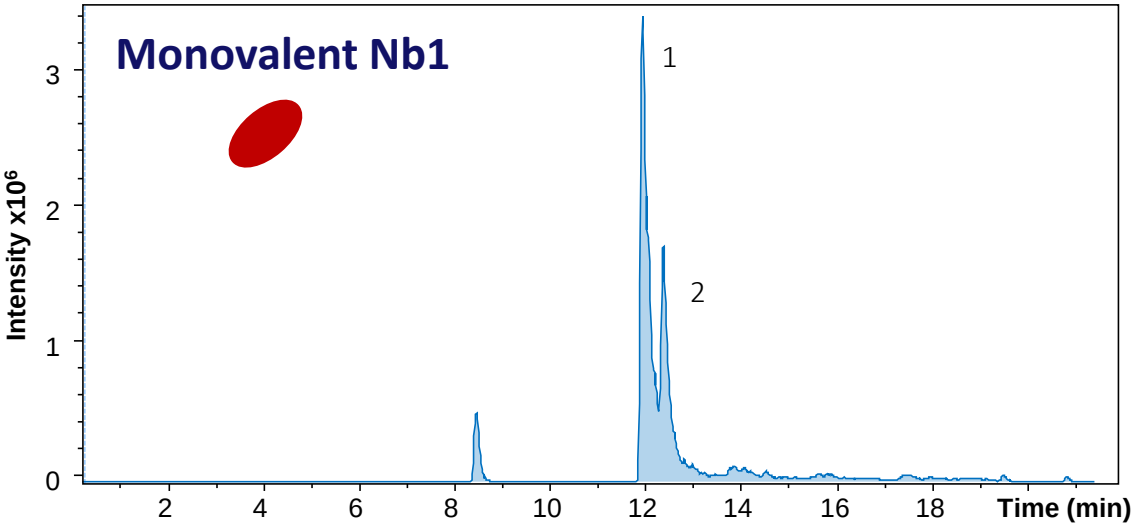
Common modifications of nanobodies:

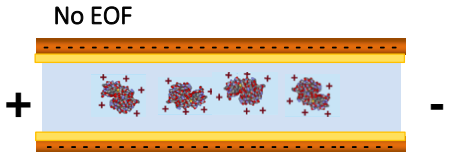
Heterogeneity
- Non-glycosylated
- PTMs, *eg.* deamidation, pyroglutamate

Degradation products
- Truncated forms (*e.g. Myc-His* tag)

Multivalent nanobodies
- Improper linkage (monovalent nanobody)

CESI-MS of nanobody-1

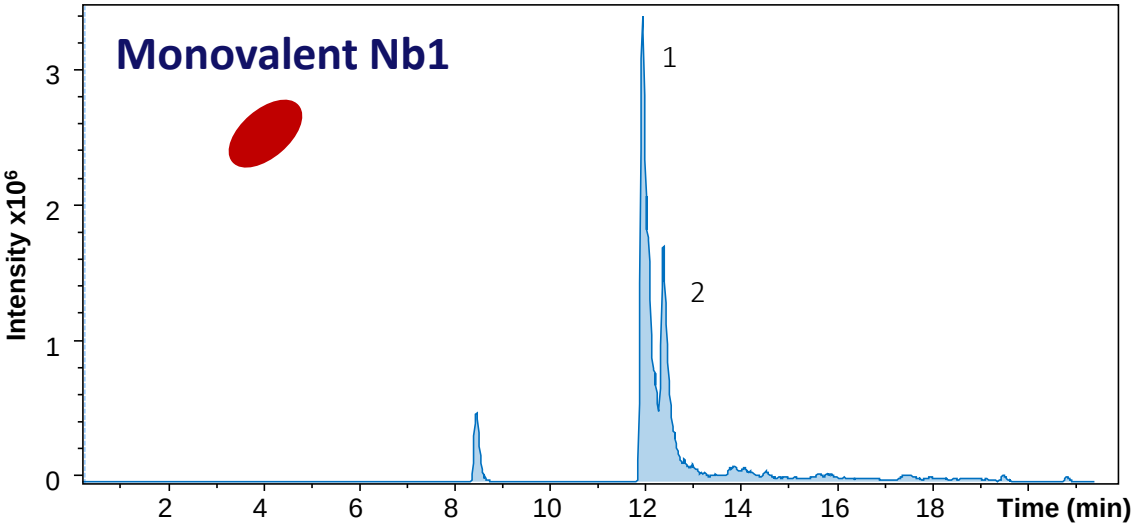


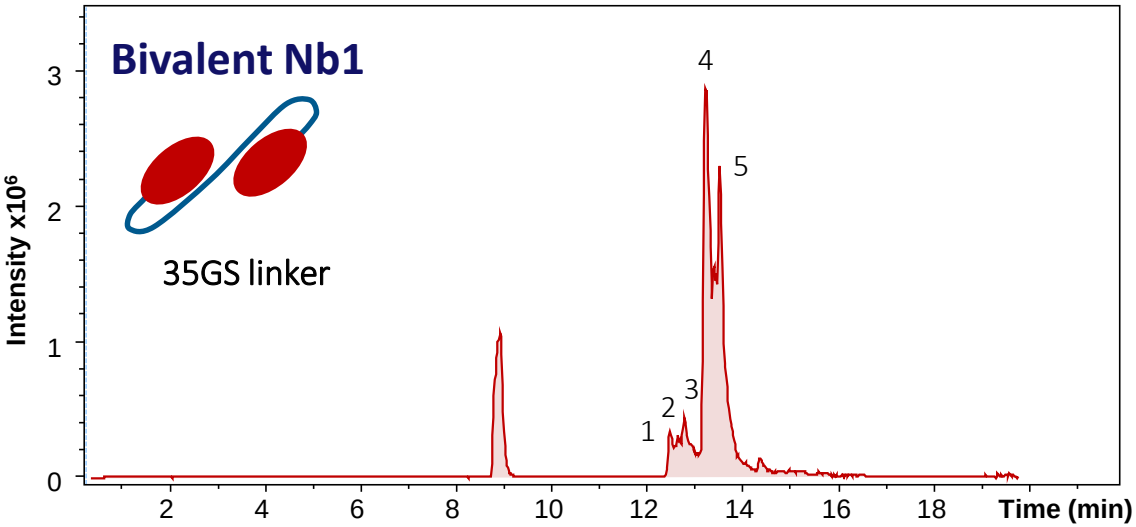


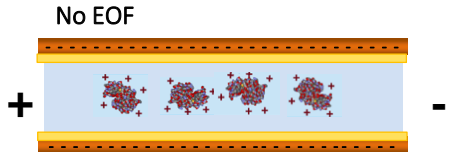
BGE, 50 mM ammonium acetate (pH 3.0).
1 µM sample (10 ng/µL)

peak	mass (Da)	assignment
1	14590.2	Nb1
2	14591.2	deamidated Nb1

CESI-MS of nanobody-1





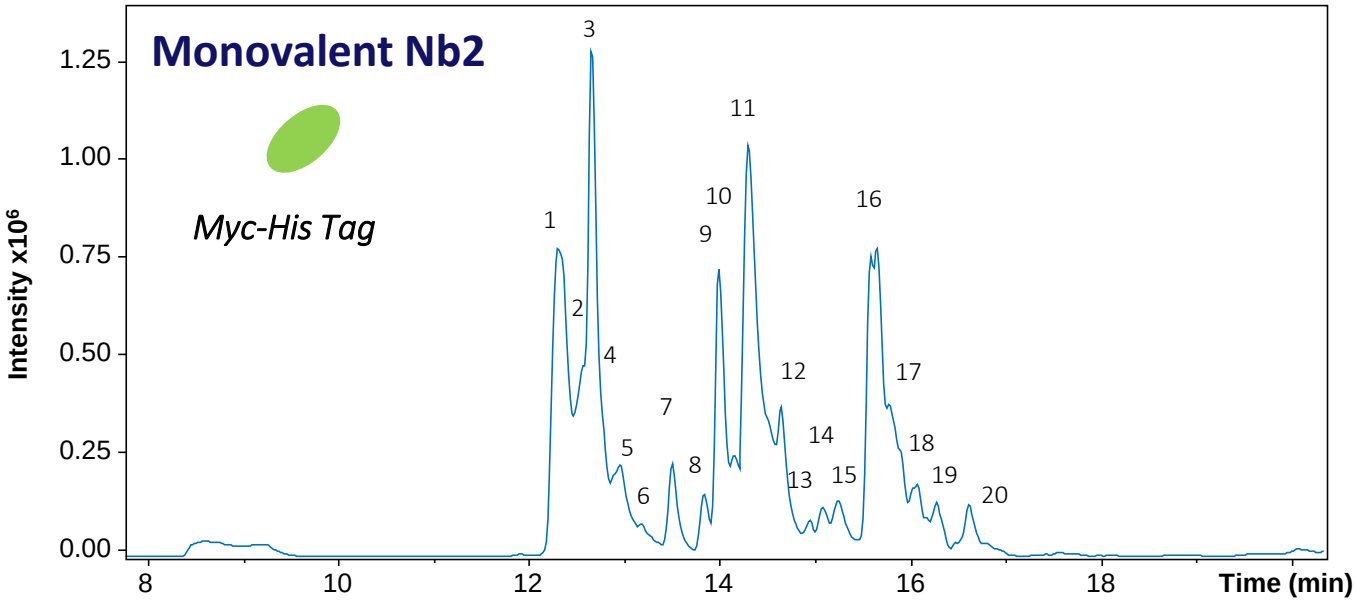


BGE, 50 mM ammonium acetate (pH 3.0).
1 µM sample (10 ng/µL)

peak	mass (Da)	assignment
1	14590.2	Nb1
2	14591.2	deamidated Nb1

peak	mass (Da)	assignment
1	14590.2	Nb1
2	14591.2	deamidated Nb1
3	15527.7	Nb1+$(GGGGS)_{3?}$
4	28835.4	Nb1-35GS-Nb1
5	28836.4	deamidated Nb1-35GS-Nb1

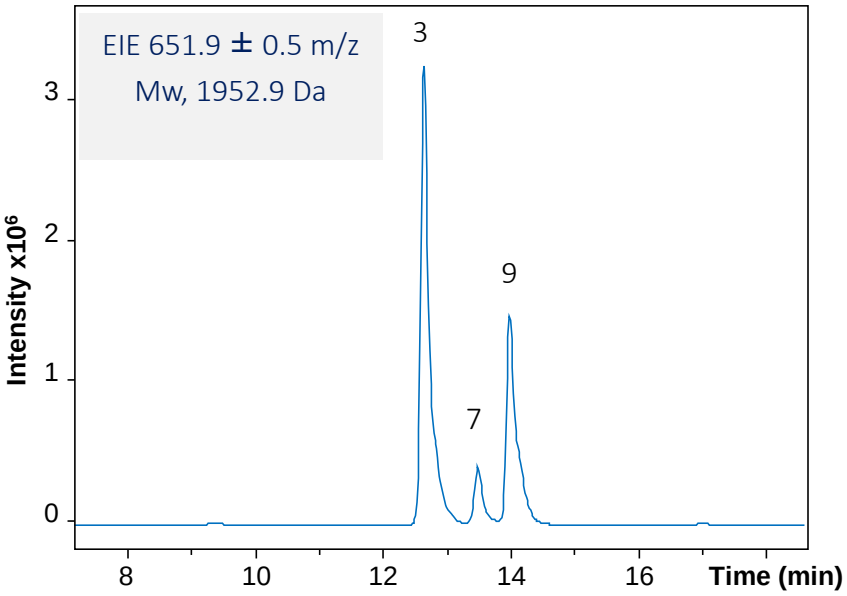
CESI-MS of nanobody-2

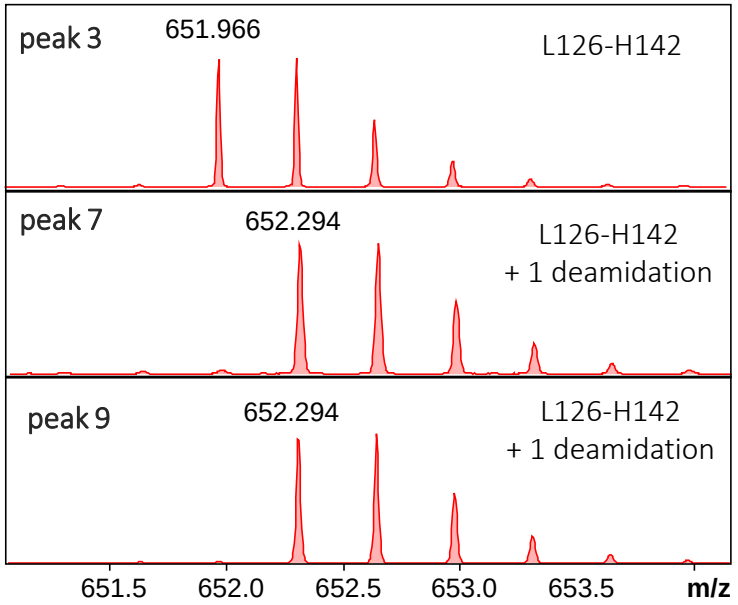


peak	mass (Da)	assignment
1	15498.5	10A10
2	15499.5	deamidated 10A10
3	1952.9	L126-H142
4	15499.5	deamidated 10A10
5	15500.5	bisdeamidated 10A10
6	15500.5	bisdeamidated 10A10
7	1953.9	deamidated L126-H142
8	15087.4	10A10-(H140-H142)
9	1953.9	deamidated L126-H142
10	15088.3	deamidated M10A10-(H140-H142)

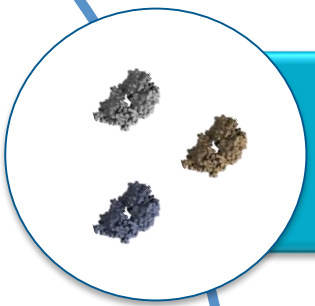
peak	mass (Da)	assignment
11	13561.6	10A10-(L132-H142)
12	13874.8	10A10-(E130-H142)
13	14004.9	10A10-(E129-H142)
14	13305.5	10A10-(Q124-H142)
15	13434.5	10A10-(K125-H142)
16	14604.2	10A10-(A136-H142)
17	14605.2	deamidated 10A10-(A136-H142)
18	14605.2	deamidated 10A10-(A136-H142)
19	14606.2	bisdeamidated 10A10-(A136-H142)
20	14606.2	bisdeamidated 10A10-(A136-H142)

Resolution of isomeric forms

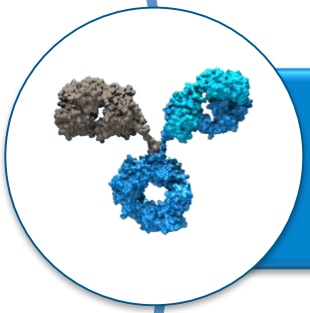




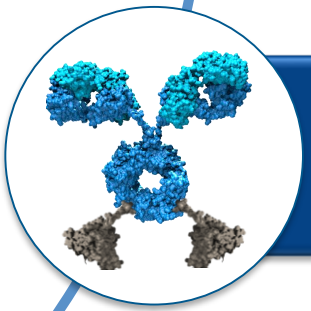
Outline



NANOBODIES



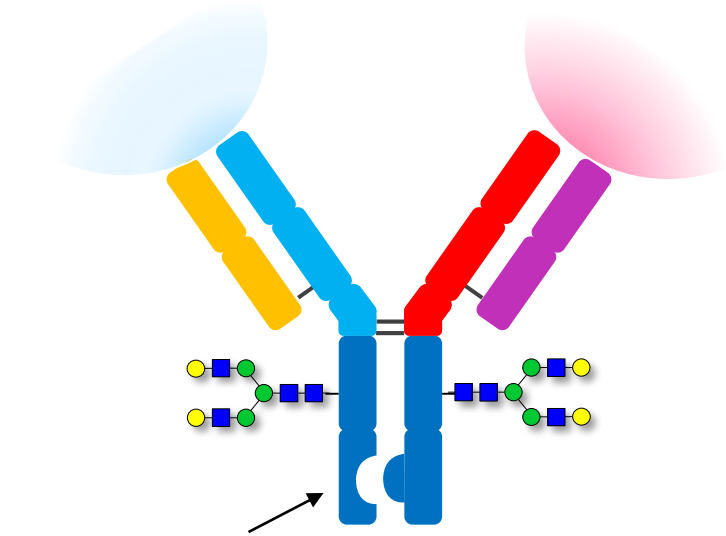
BISPECIFIC ANTIBODIES



FUSION PROTEINS

Bispecific antibodies

two different binding sites
(different targets)



Complementary CH3
mutation

Common side products

Heavy Chain dimer

¾ antibody

half antibodies

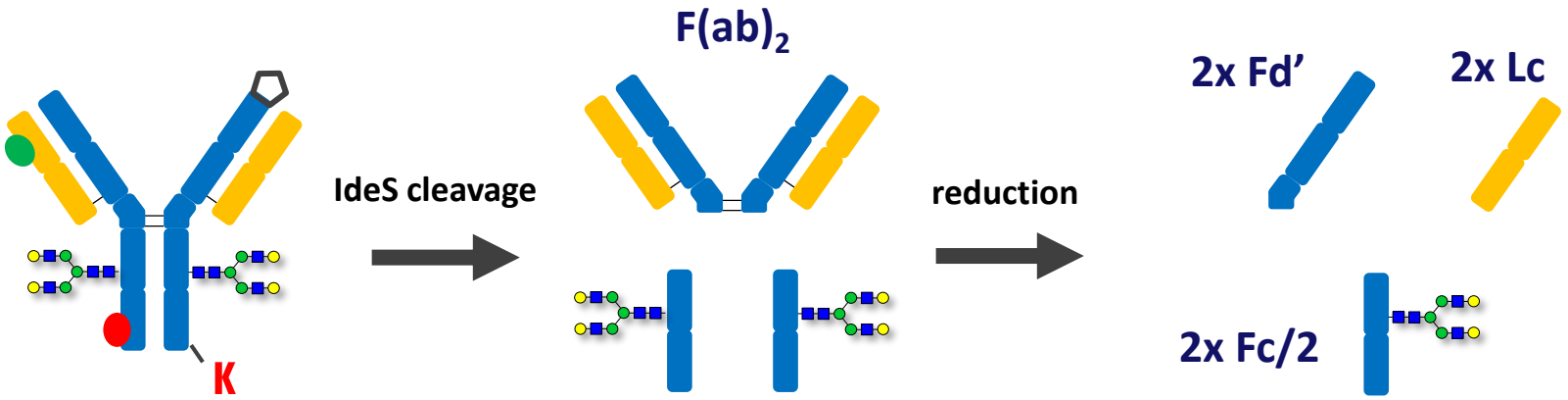
free LC and LC dimers
(LC Homo- and Heterodimers)

anti-Target 1 antibody

anti-Target 2 antibody

Looking at microheterogeneity: Middle-up approach

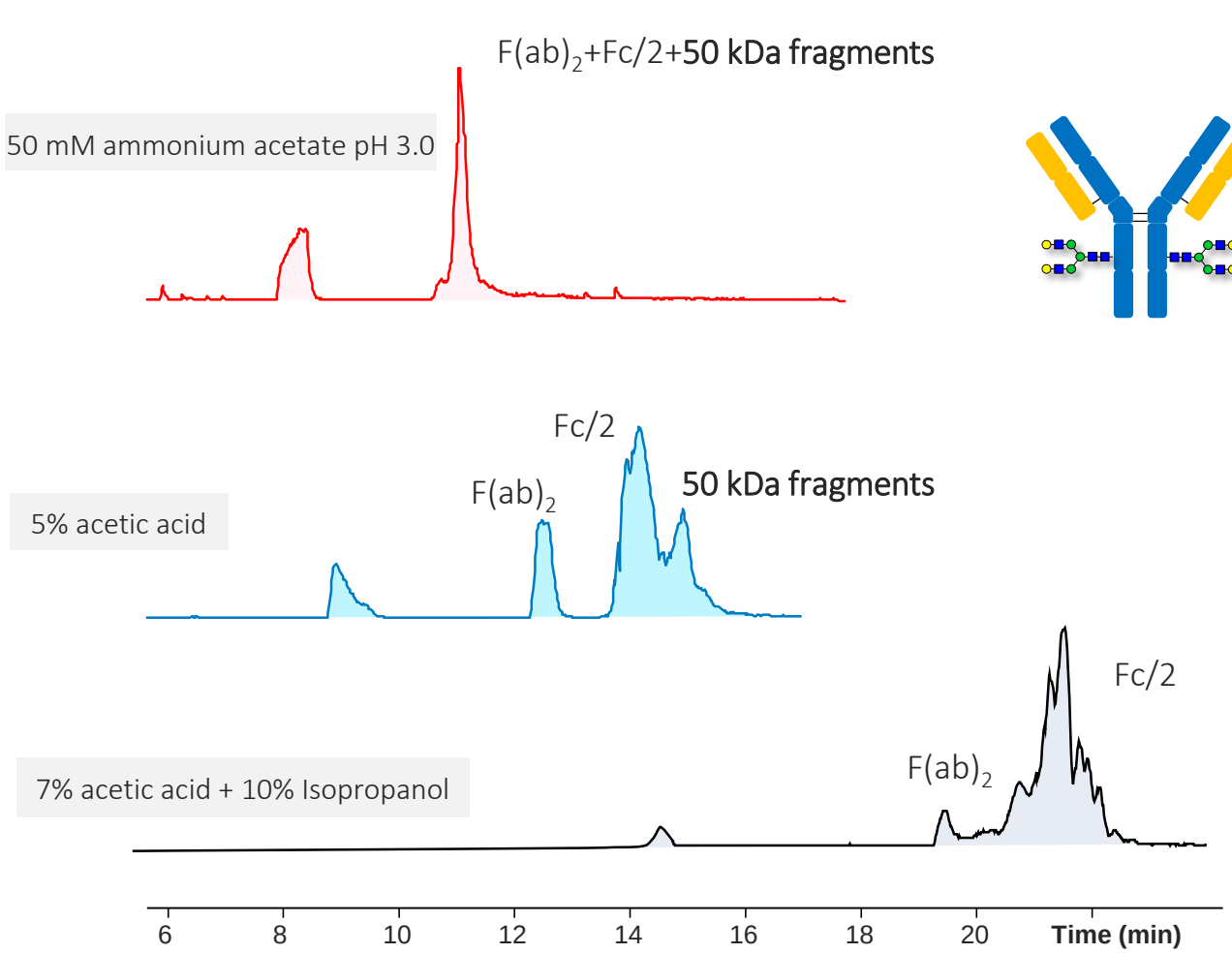
Middle-up approach: Domain specific analysis of microheterogeneity

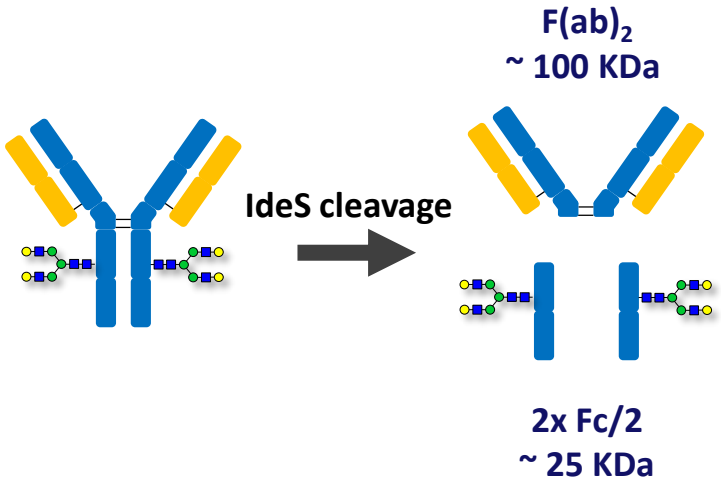


Monospecific mAb

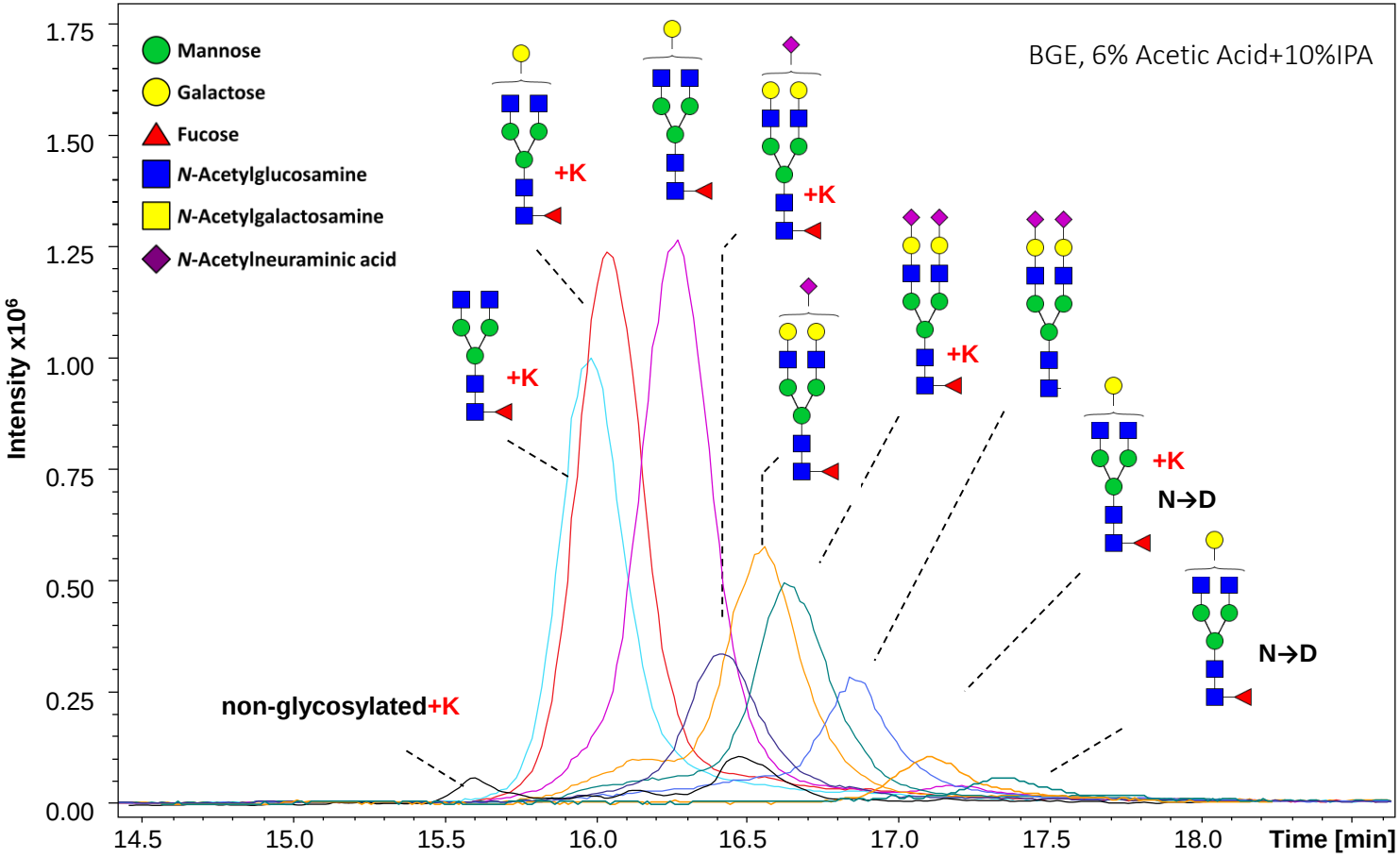
2x Fc/2

CESI-MS of digested monoclonal antibodies



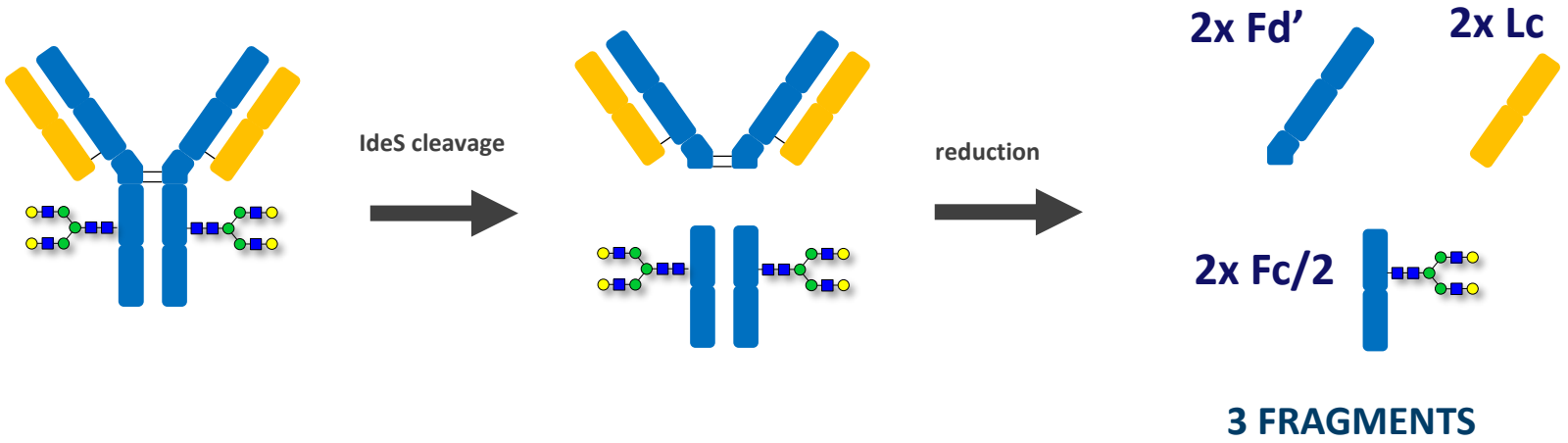


Charge heterogeneity of Fc/2 fragments



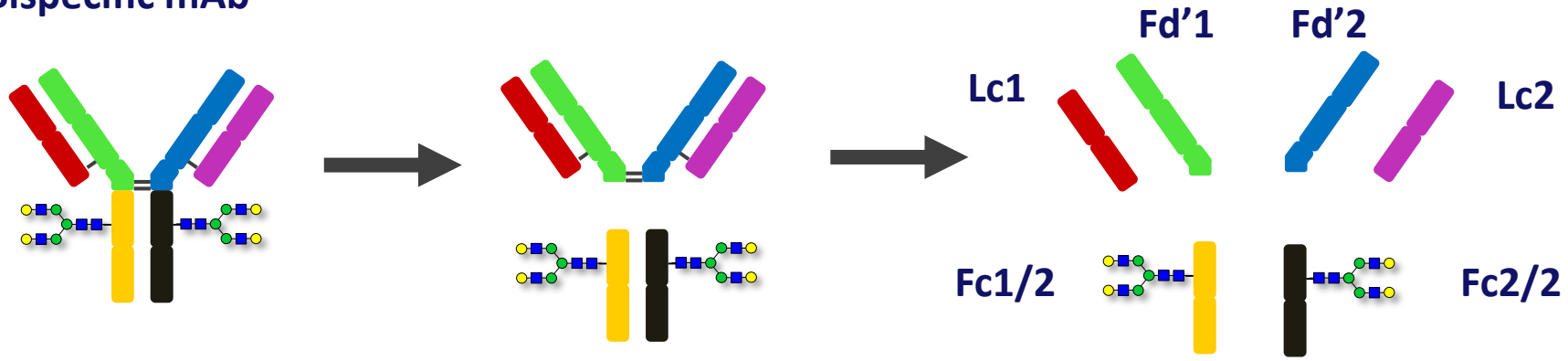
Middle-up of bispecific antibodies

Monospecific mAb



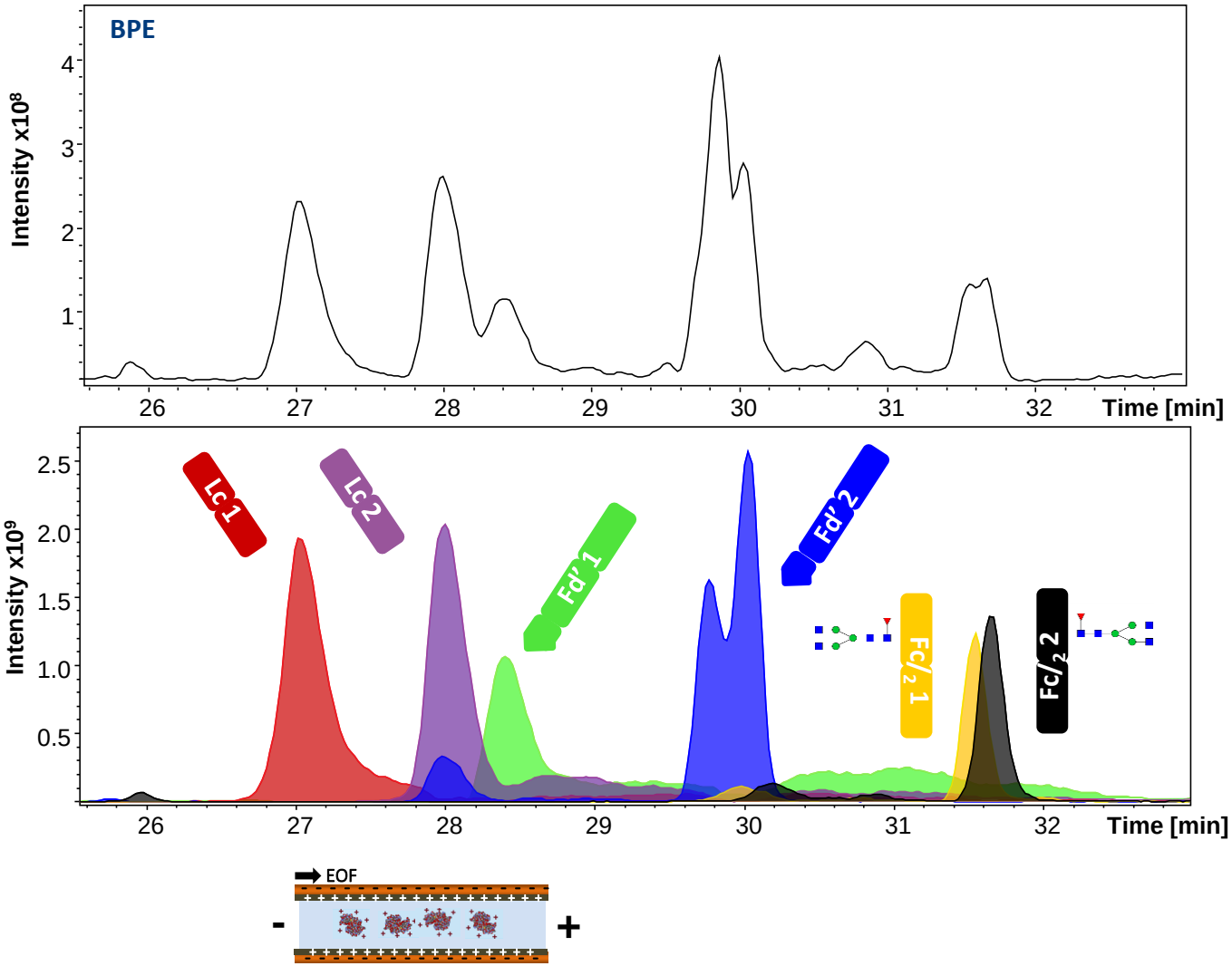
3 FRAGMENTS

Bispecific mAb

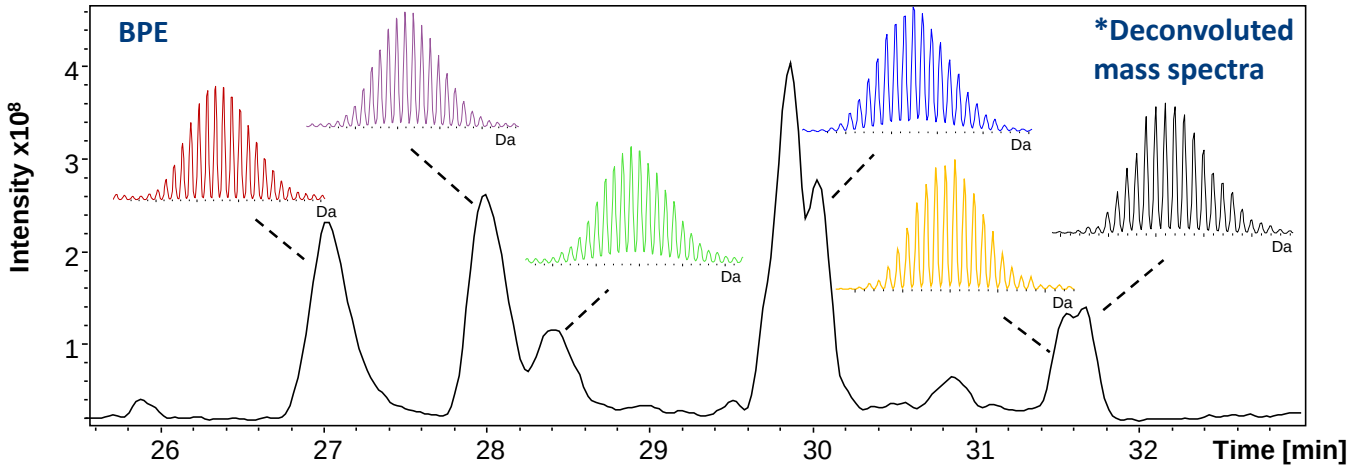


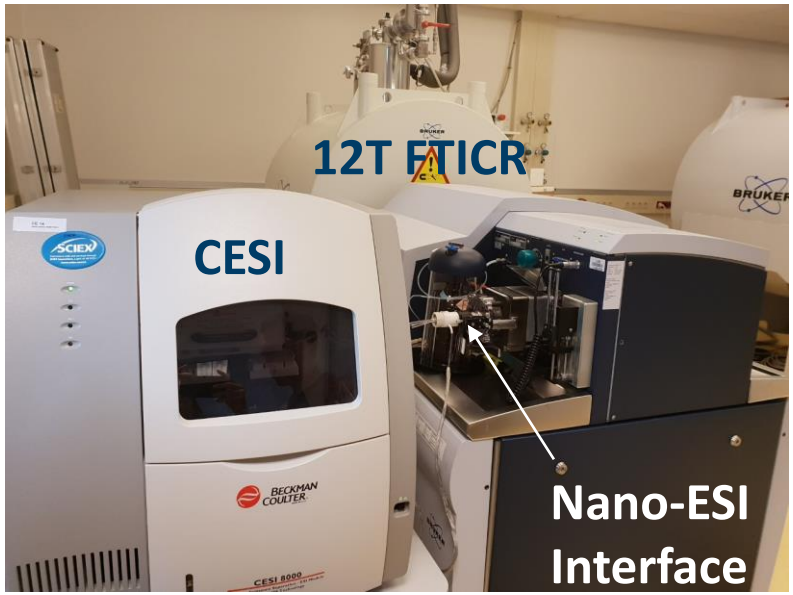
6 FRAGMENTS

CE-FTICR-MS of a bispecific mAb

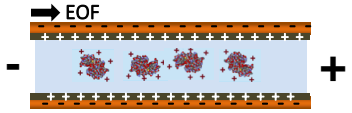


CE-FTICR-MS of a bispecific mAb

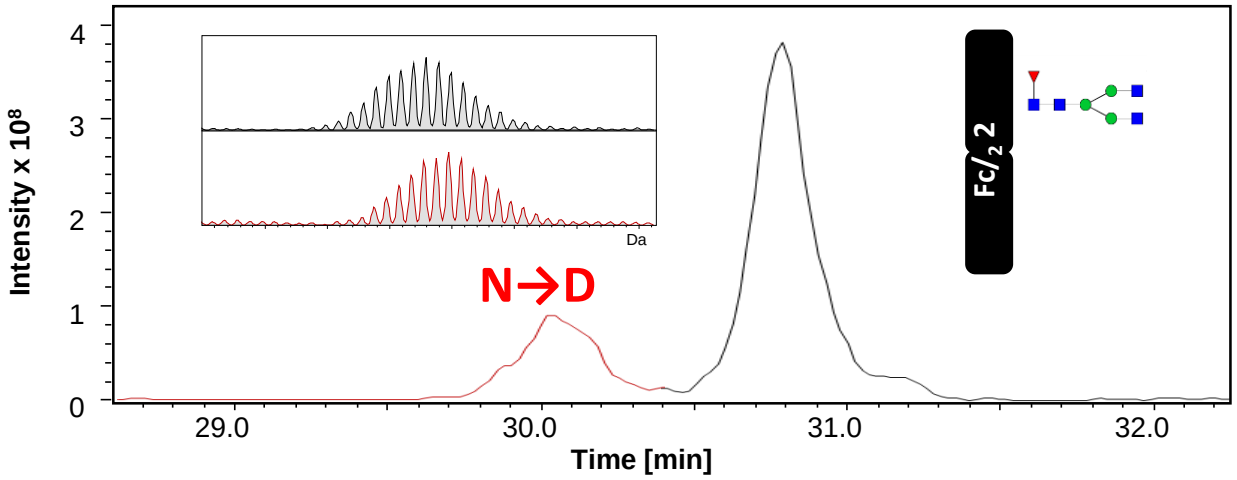


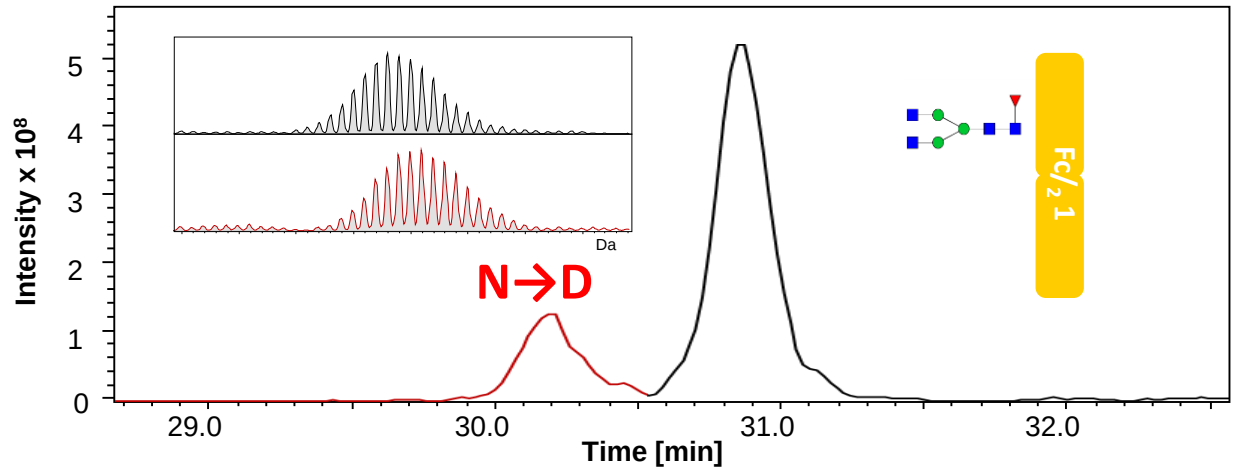


CE-FTICR-MS of a bispecific mAbs

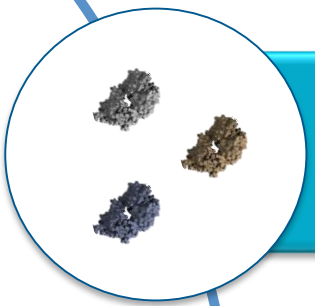


BGE, 10% Acetic Acid + 20% IPA
Separation, -20 kV, 20 °C

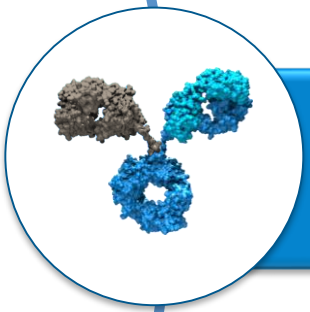




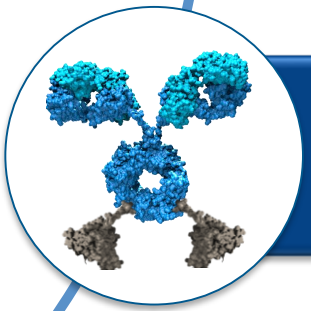
Outline



NANOBODIES



BISPECIFIC ANTIBODIES

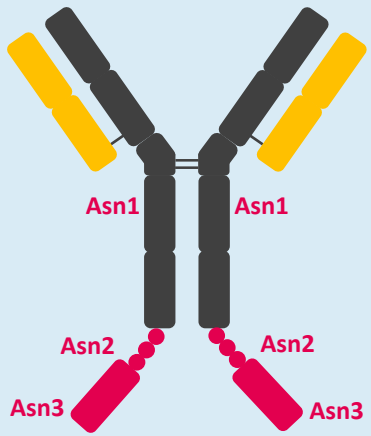


FUSION PROTEINS

Fusion protein with multiple glycosylation sites

New biological entity (NBE)

Bifunctional fusion protein

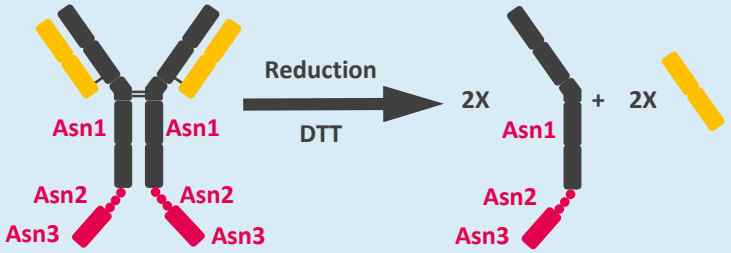


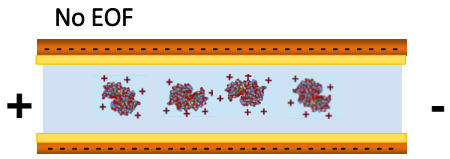
- 6 potential glycosylation sites
- Elongated heavy chain (> 180 kDa)
- Reduction of -S-S- bridges results in two main fragments
  - Fragment A: non glycosylated
  - Fragment B: 3 potential glycosylation sites, Asn1, Asn2 and Asn3
    - Asn1 and Asn2 fully occupied
    - Asn3 occupancy 50%

CESI-MS of protein with multiple glycosylation sites

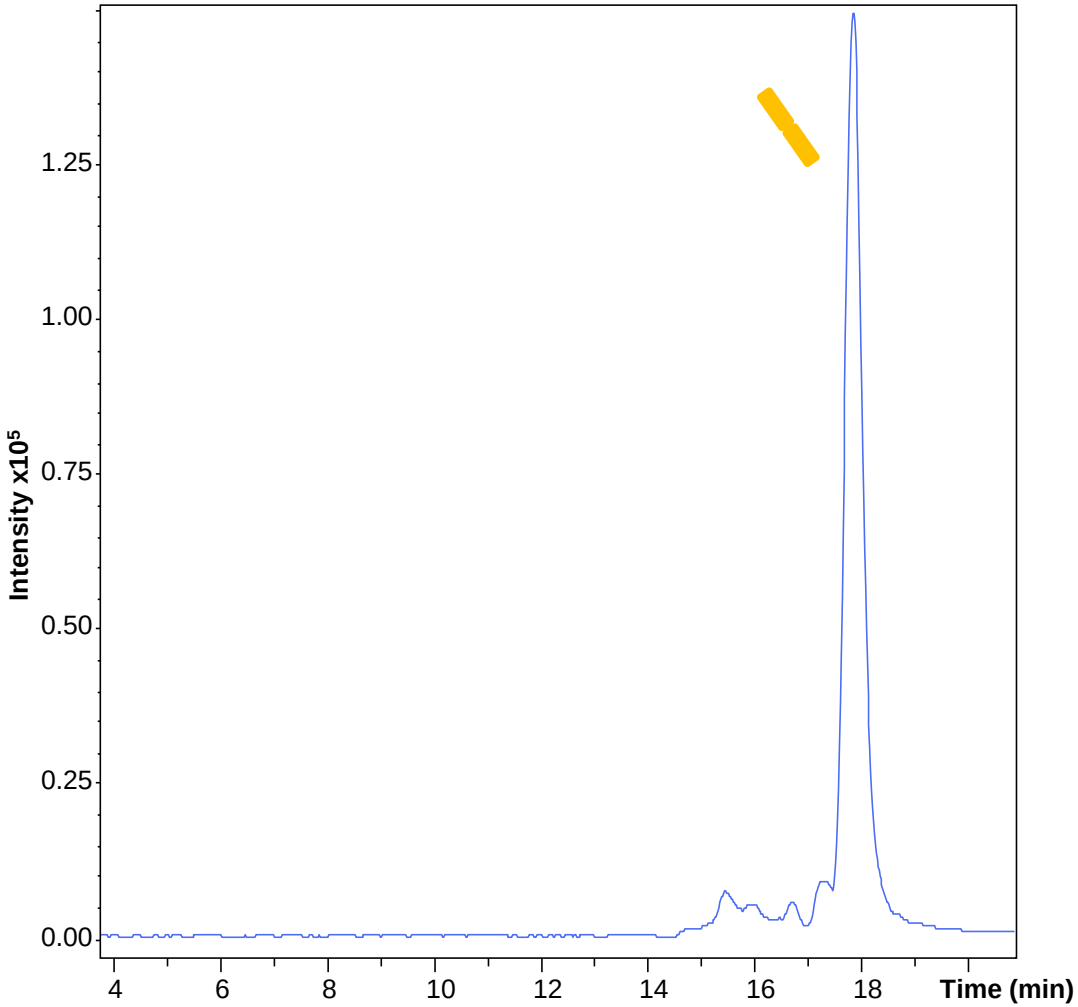
New biological entity (NBE)

Bifunctional fusion protein





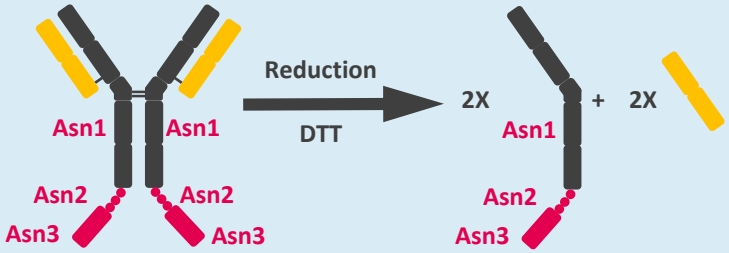
BGE, 10% Acetic Acid

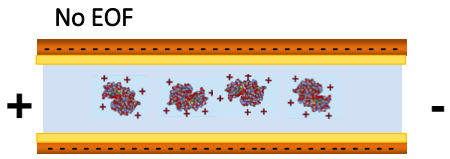


CESI-MS of protein with multiple glycosylation sites

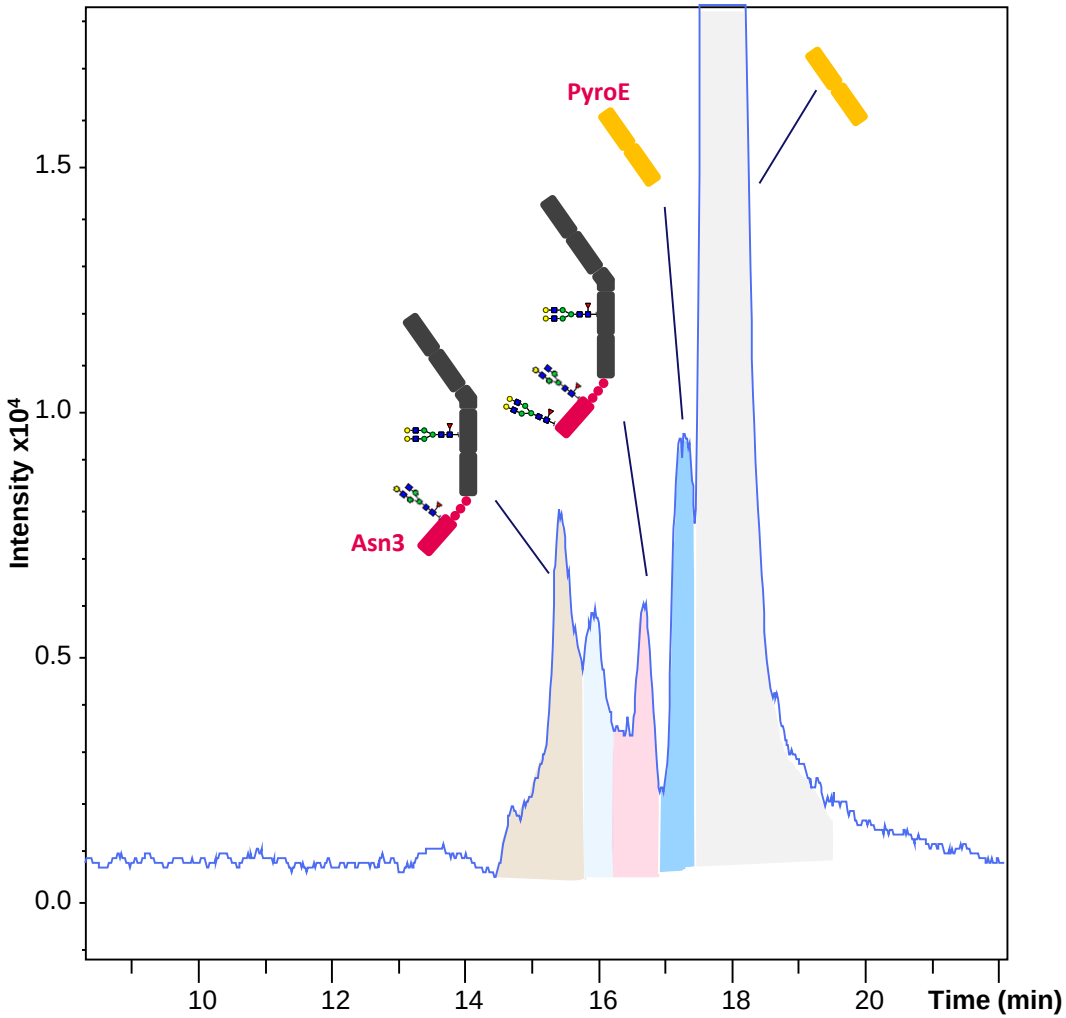
New biological entity (NBE)

Bifunctional fusion protein

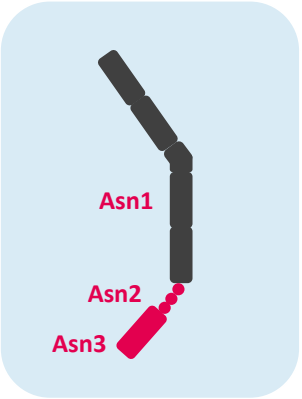


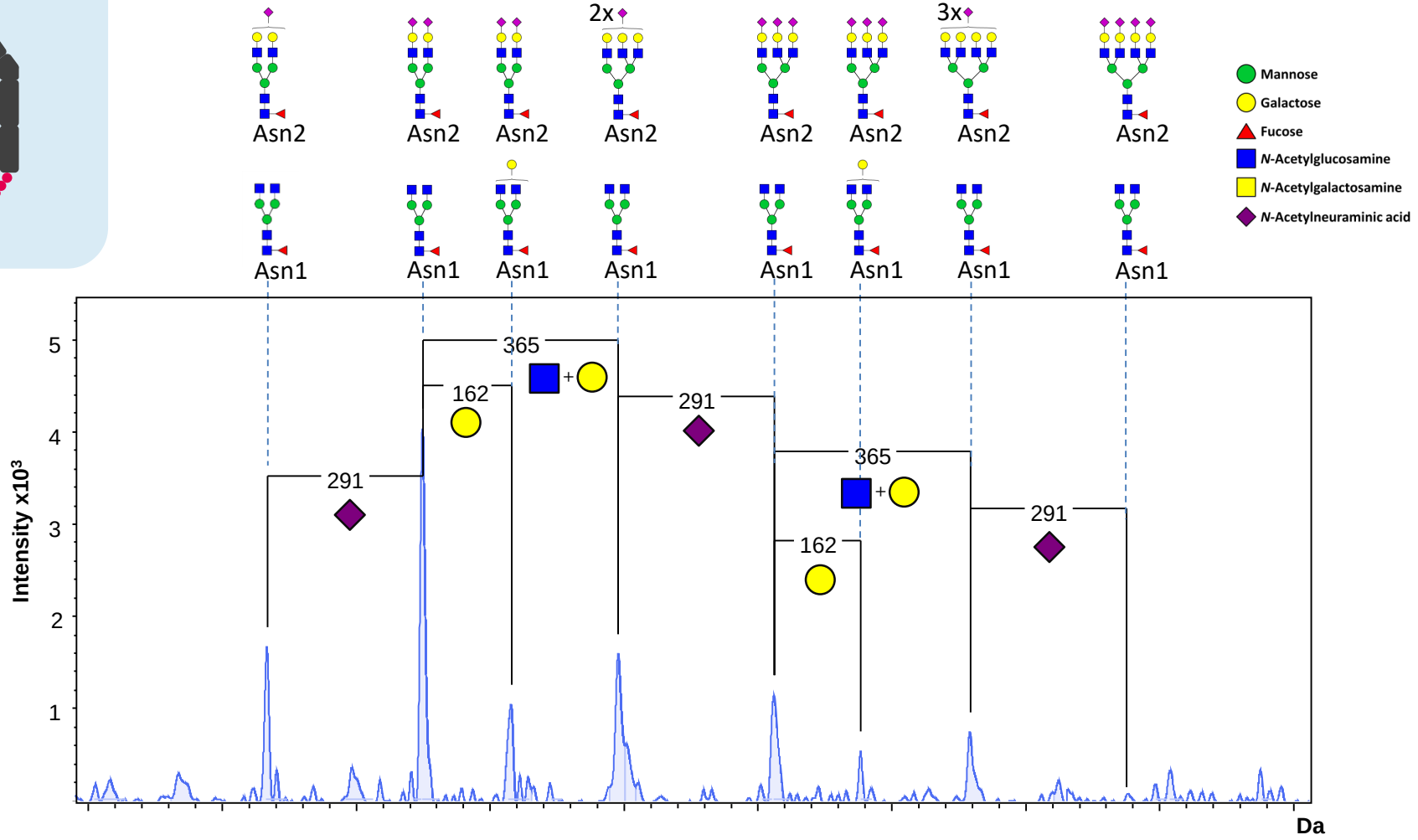


BGE, 10% Acetic Acid

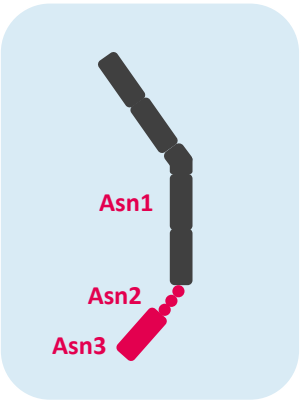


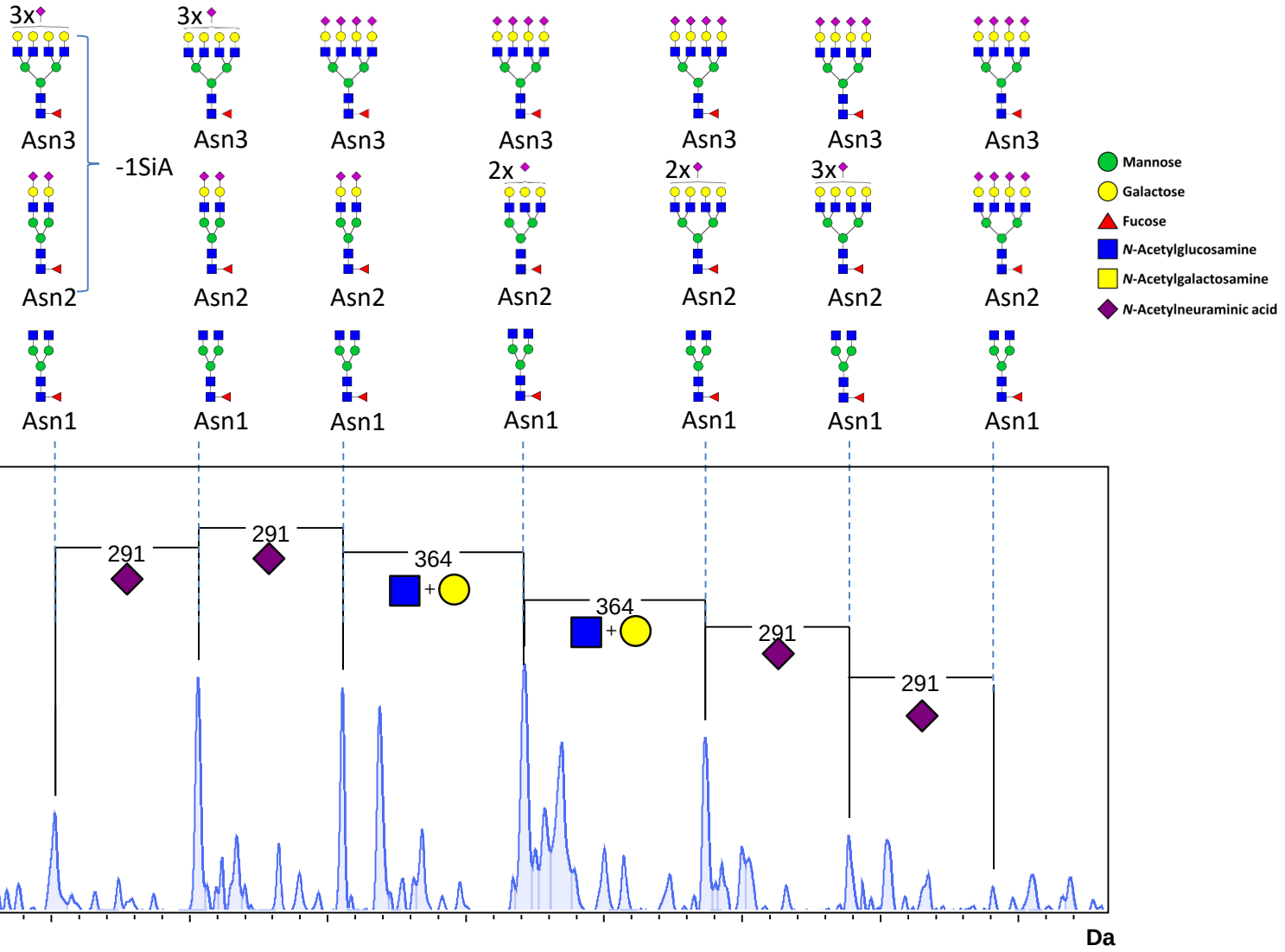
Glycoforms containing glycans at Asn1 and Asn2





Glycoforms containing glycans at Asn1, Asn2 and Asn3





Conclusions

Sheathless CE-MS allows characterization of different new antibody-derived therapeutics

- efficient protein separations
- sensitive detection
- suitable for proteins with very different characteristics
- assessment of micro and macroheterogeneity

Perspectives

- *study of antibody interactions*
- *analysis of human immunoglobulins*

Leiden University Medical Center

Acknowledgements

CPM – Center for Proteomics and Metabolomics

CPM – Glycomics and Glycoproteomics

Christoph Gstöttner

Steffen Lippold

Simone Nicolardi

Manfred Wuhrer

VU University Amsterdam

BioAnalytical Chemistry

Rob Haselberg

Govert W. Somsen

Medicinal Chemistry

Raimond Heukers

Martine Smit

SCIEX

A4B Analytics for Biologics