

HOW TO USE PROTEIN SPECIFIC RETENTION BEHAVIOR TO IMPROVE THE CHARACTERIZATION OF THERAPEUTIC ANTIBODIES

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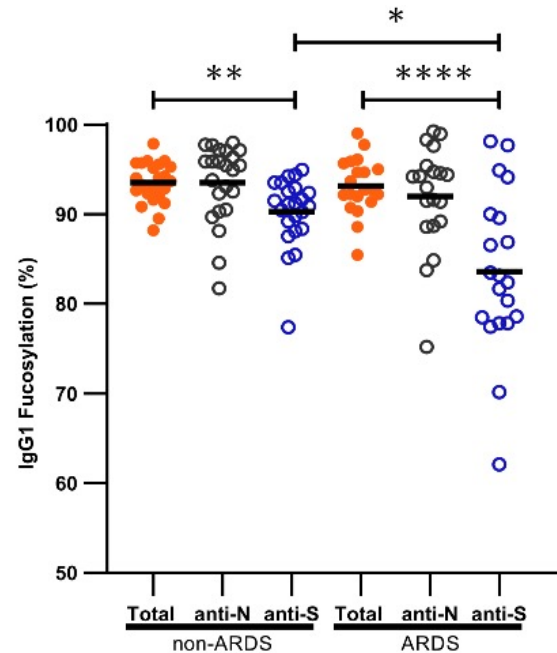
Importance of antibodies in the COVID-19 health crisis

Antibody self-testing for COVID-19 infections



Quick serology test for COVID-19 using a chromatographic immunoassay

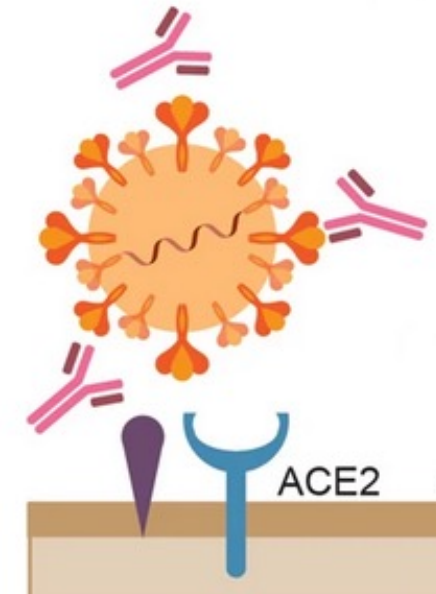
Pathophysiological differences for COVID-19 antibodies



Decreased fucosylation for critically ill (ARDS) patients triggering excessive inflammations

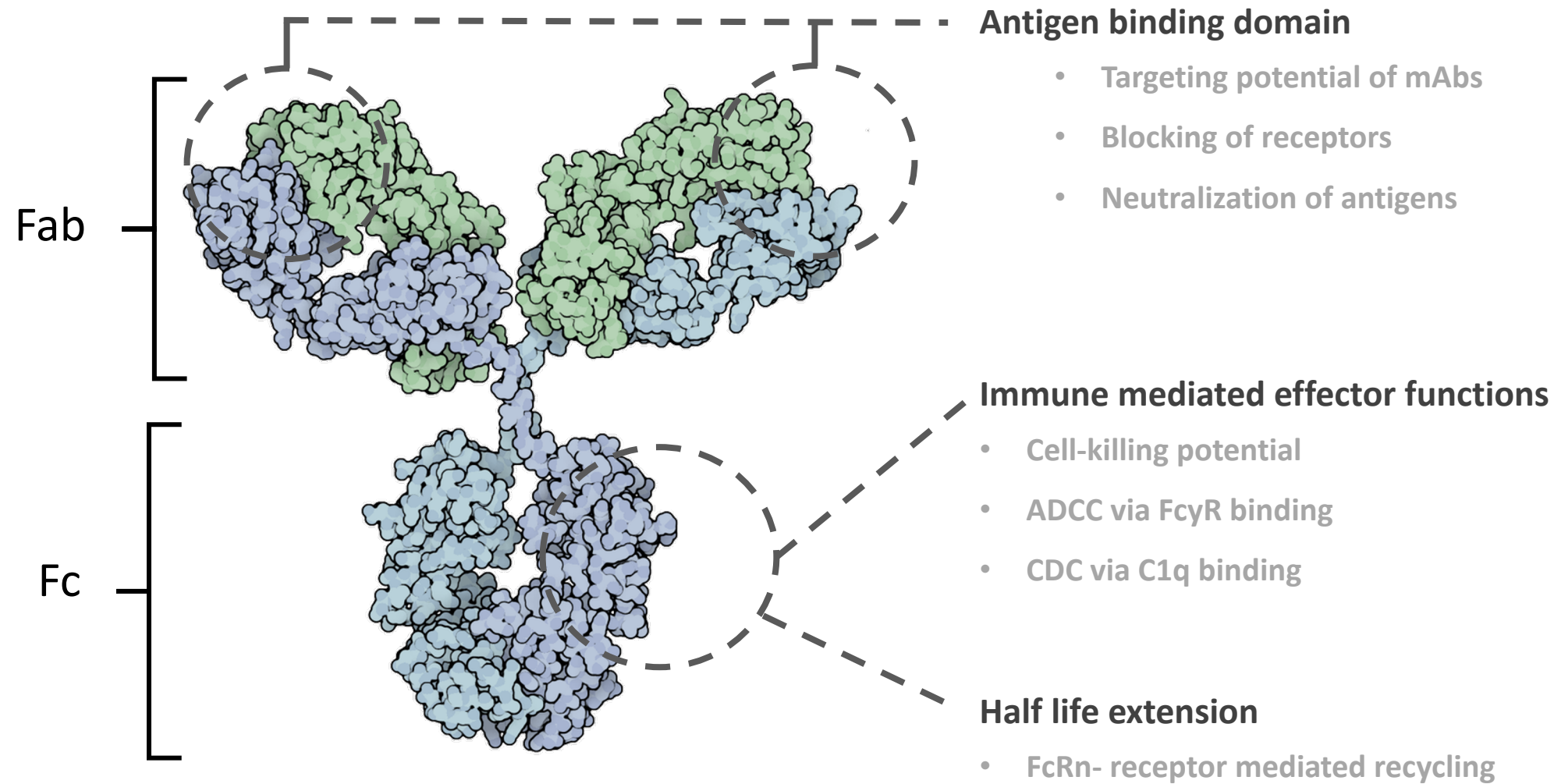
Larsen et al. (2021) Science

Therapeutic antibodies to treat infected patients



Blocks spreading of the virus in the body and prevents hospitalization of high-risk patients

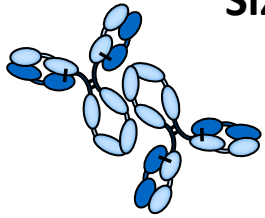
Benefits of using therapeutic monoclonal antibodies



Antibodies are ~ 800 times larger than paracetamol!

Complex characterization of mAb-based products

Size-variants

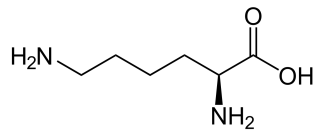


Aggregation

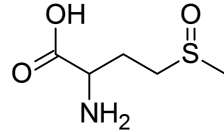


Fragmentation

Charge variants

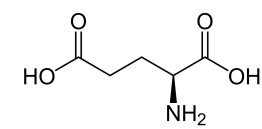


Lys glycation

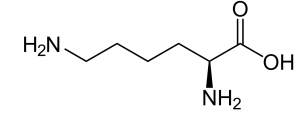


Met oxidation

Site-specific

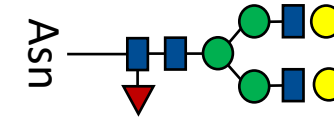


N-terminal pyroGlu formation



C-terminal lysine clipping

N-Glycosylation



● = Galactose

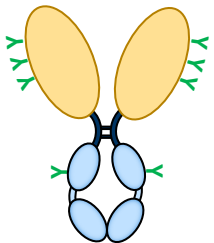
● = Mannose

■ = N-Acetyl Glucosamine

▲ = Fucose

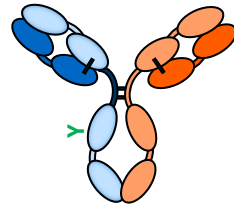
Fusion proteins

N/O-Glycosylation of the fused partner



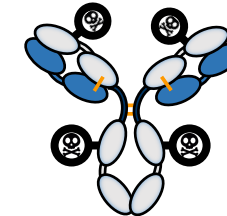
Bispecific mAbs

Chain specific modifications
Correct heterodimerization



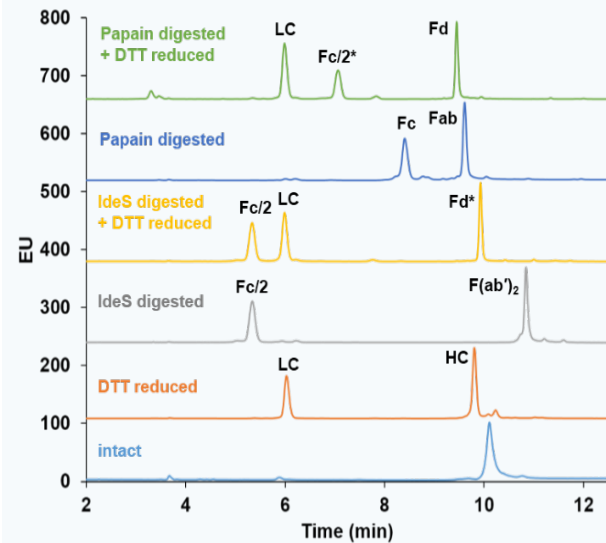
Antibody-Drug Conjugates

Drug load distribution (DLD)
Drug to antibody ratio (DAR)

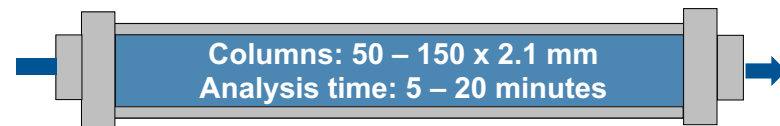


Chromatography provides an important tool for characterization

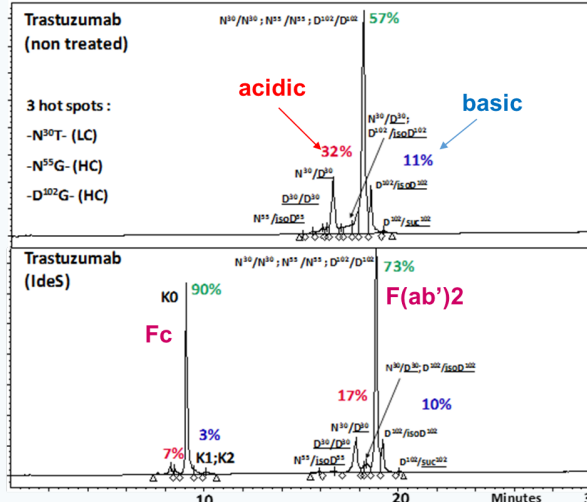
I. Reversed Phase (RP)



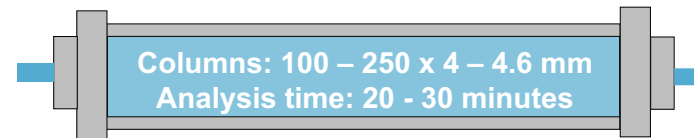
Intact and subunit proteins



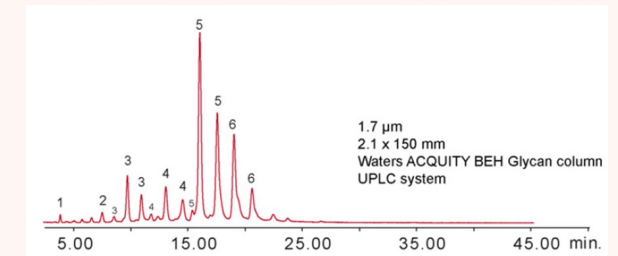
II. Ion-Exchange (IEX)



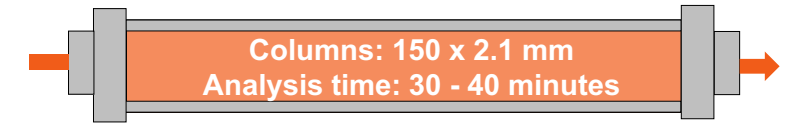
Intact and subunit proteins



III. Hydrophilic Interaction (HILIC)



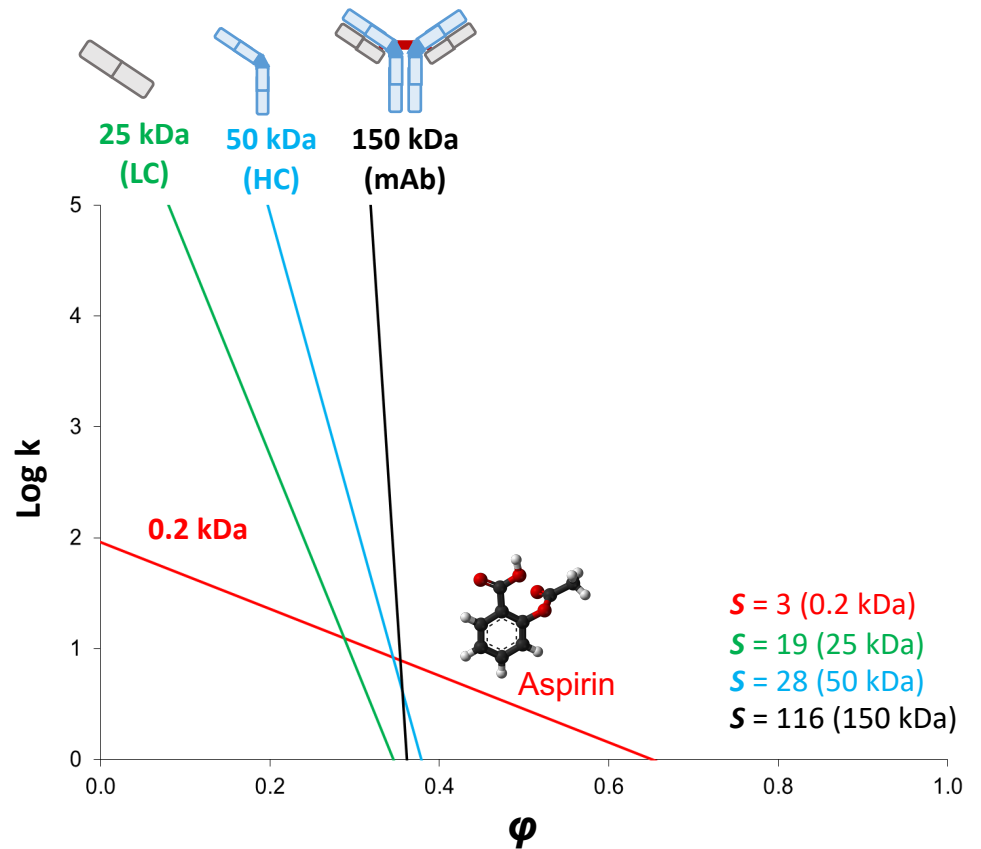
Released glycan analysis
on standard pore stationary phases



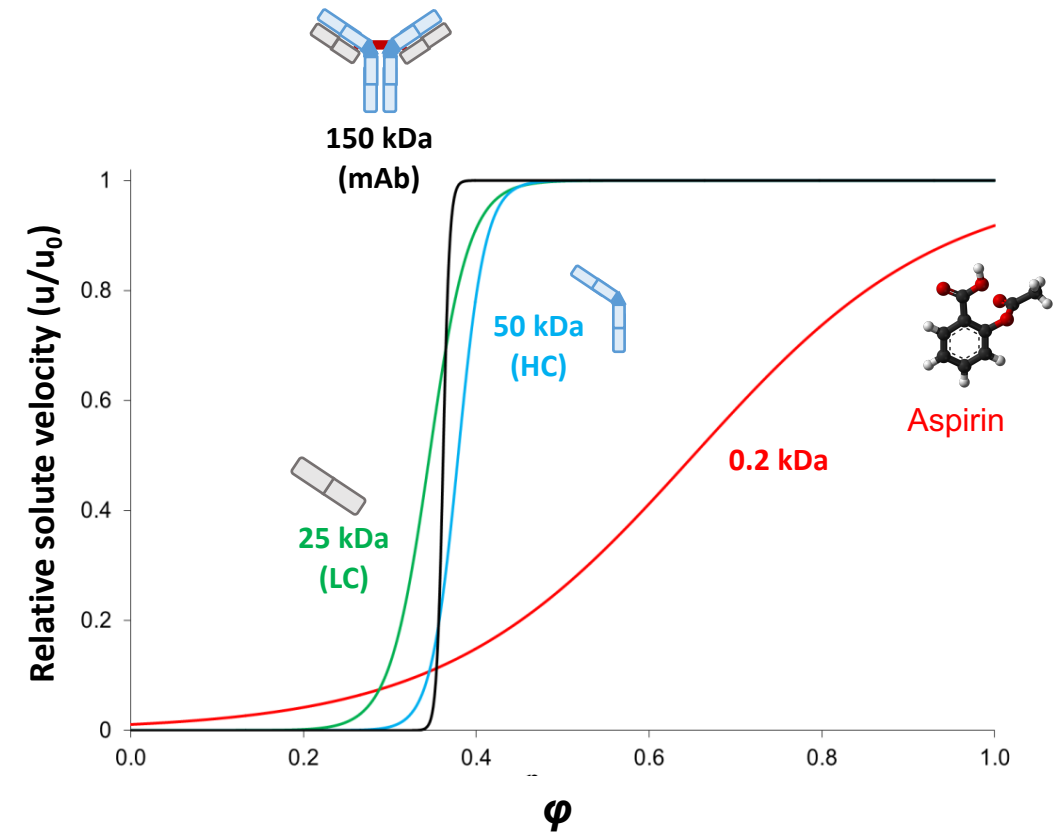
All use long linear gradients and column equilibration times!

Speeding-up by using protein-specific elution behavior

→ Using the **ON-OFF** or **Bind-and-elute** retention mechanism in **RPLC**

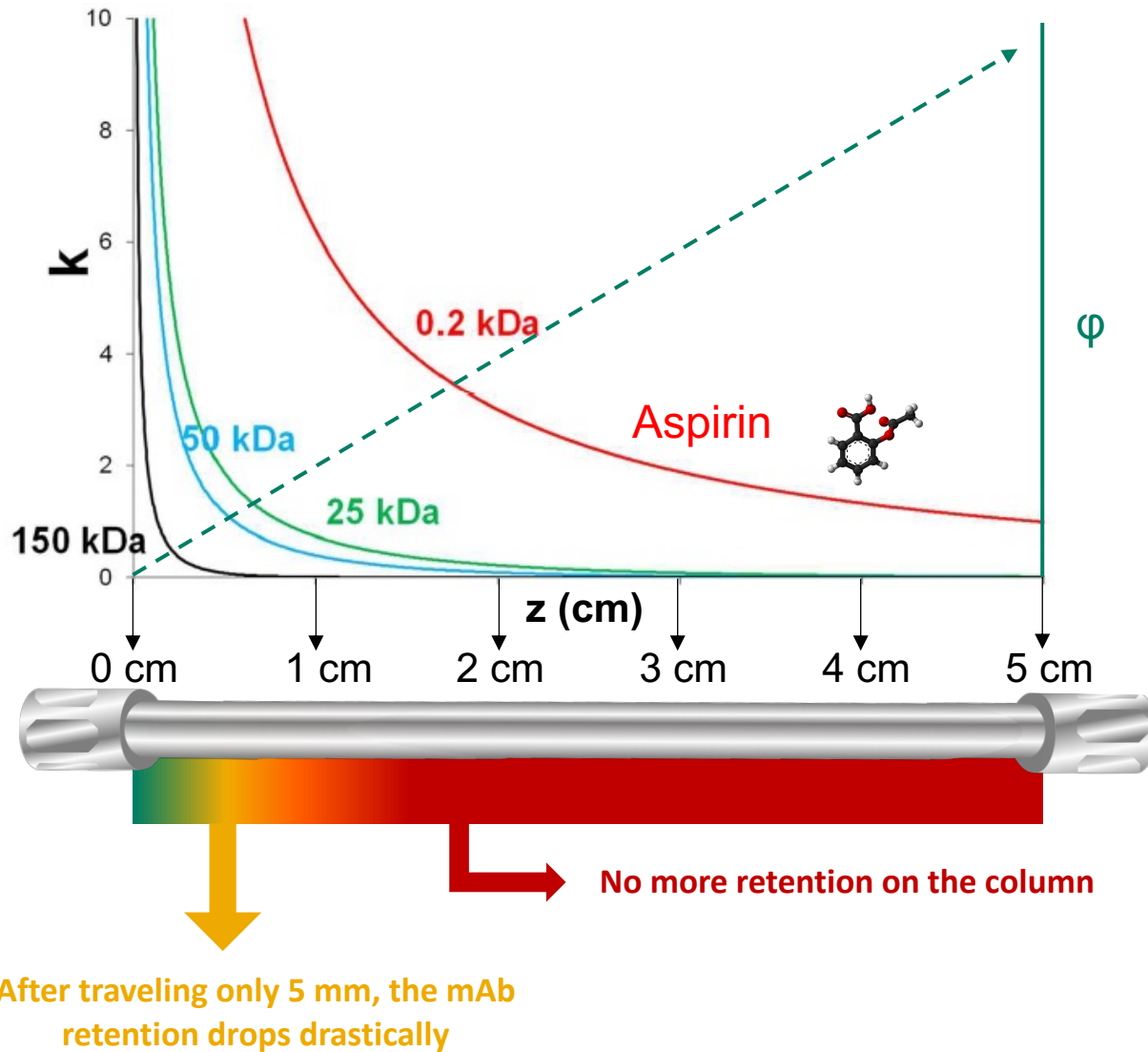


For intact mAb, $k \approx 100$ (34% ACN) and $k \approx 1$ (36% ACN)



For intact mAb, the “on” and “off” states correspond to a %ACN range of only 3.5% (37.9 - 34.4%)

Changing to more appropriate column hardware

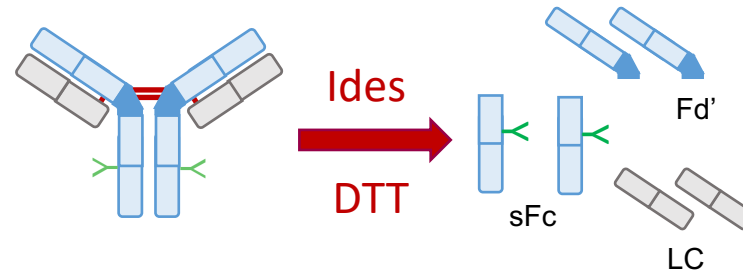


→ Ultrashort columns can be used to significantly reduce the analysis times (<1 min)

Application to RPLC analysis of anti-COVID therapeutics



Sample preparation



Experimental parameters



Bioresolve RP mAb Polyphenyl
(50 x 2.1mm, 2.7 μ m, 450 Å)

Acquity UPLC H-Class system

Sample 1 mg/mL diluted in water

MPA 0.1%DFA in water

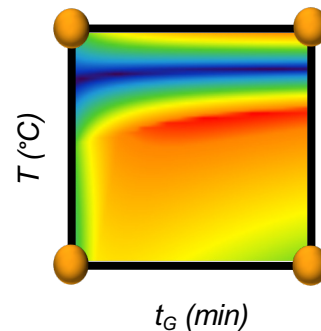
MPB 0.1% DFA in ACN

25-45%B

$F = 0.6$ mL/min

FLD detection

DOE method parameters

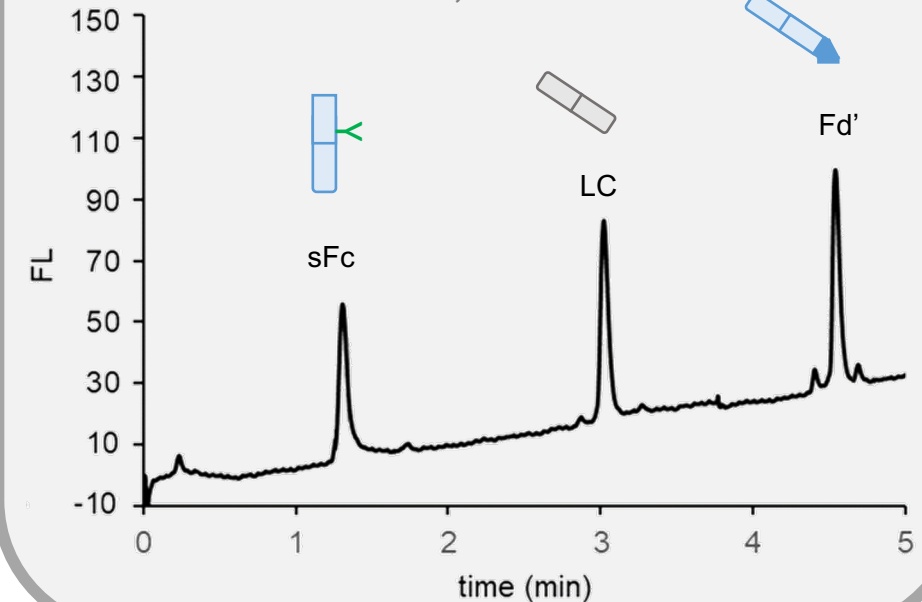


Gradient time: 4, 12 min

Temperature: 65, 90 °C

Imdevimab

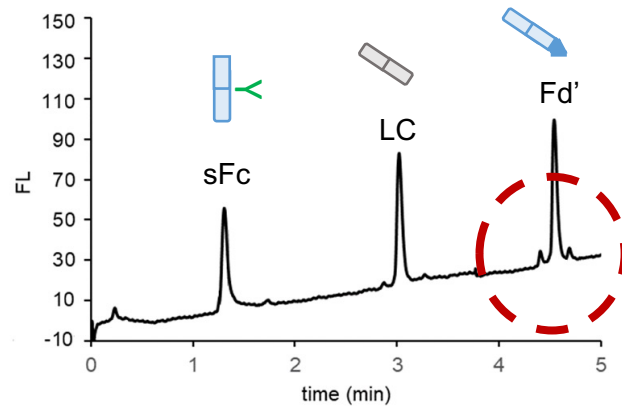
75 °C, 26–36 %B



From optimized conditions, virtually transferring to ultra-short columns

Experimentally measured on 2.1 x 50 mm column

Imdevimab digested and reduced sample



Important parameters when Transferring methods:

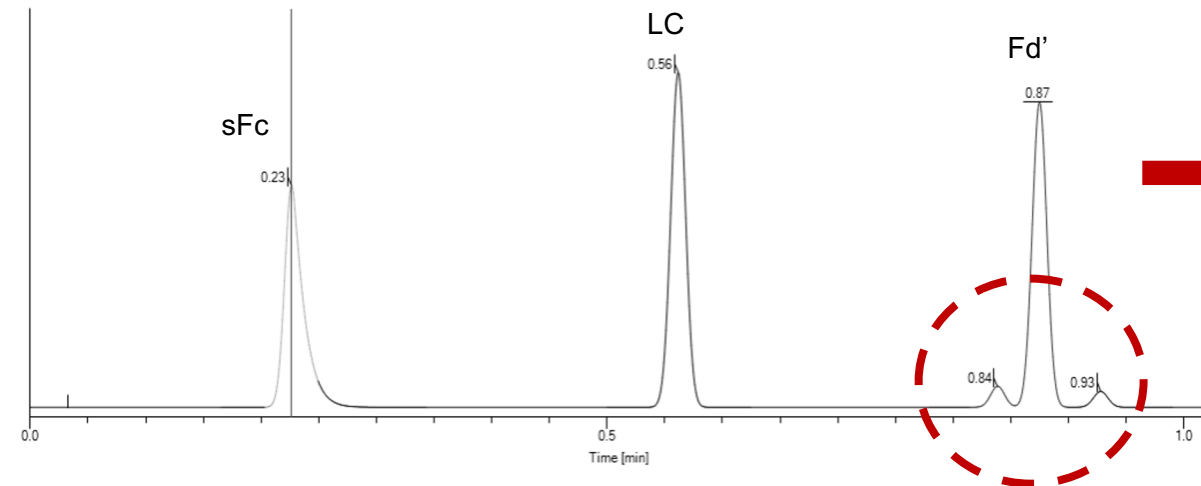
- Switch to low dwell and extra column volumes
- Change to ultrashort column parameters
- Change flow rate and gradient



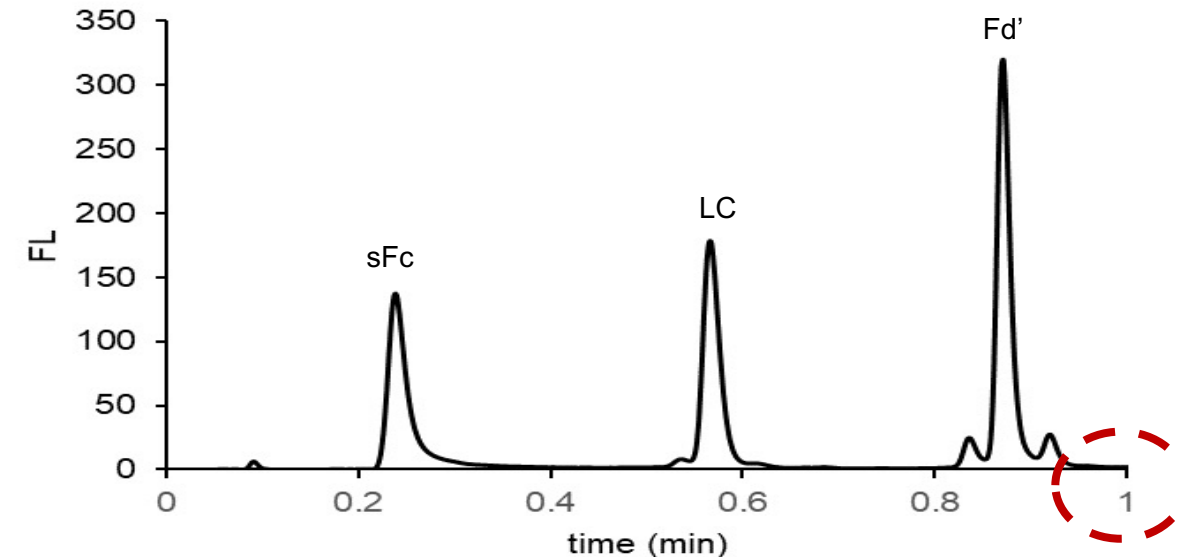
Software: Drylab, Chromsword, etc.



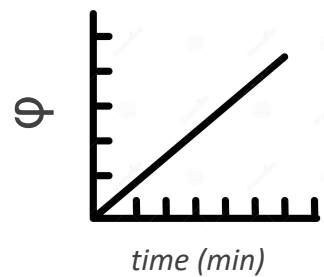
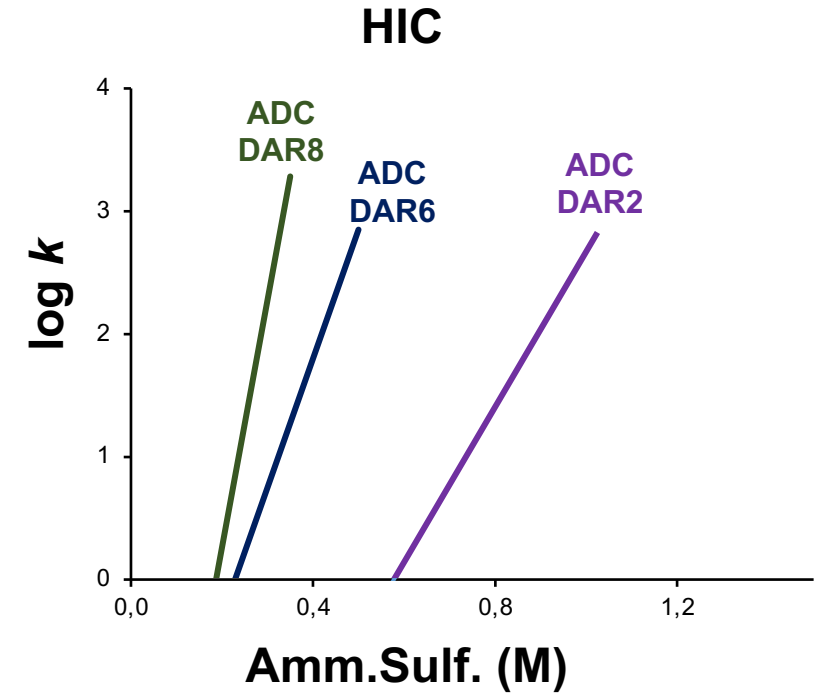
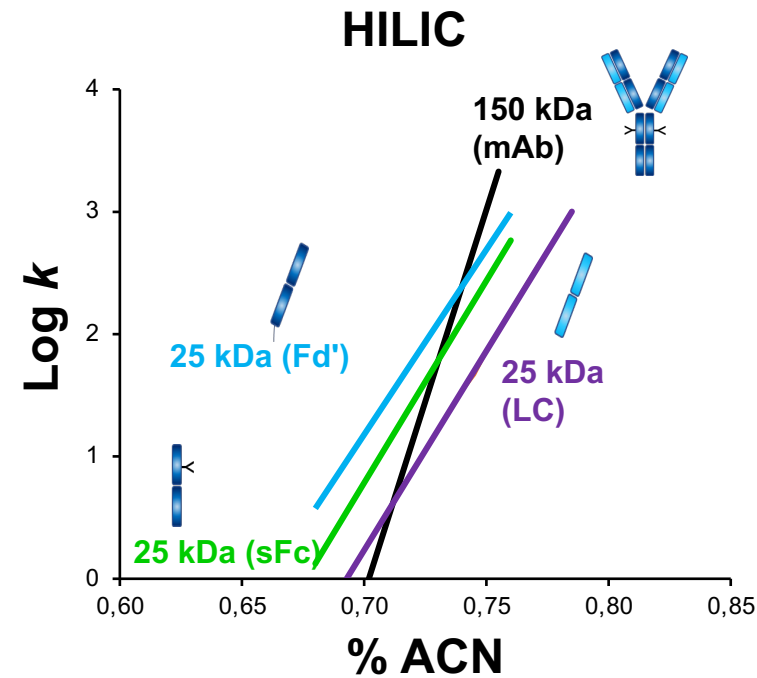
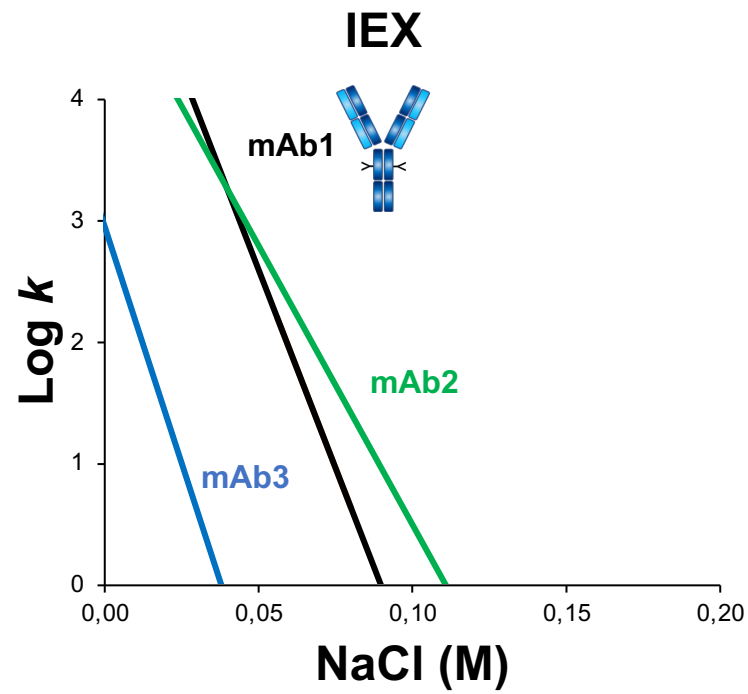
virtually transferred to 2.1 x 20 mm



experimentally measured on 2.1 x 20 mm



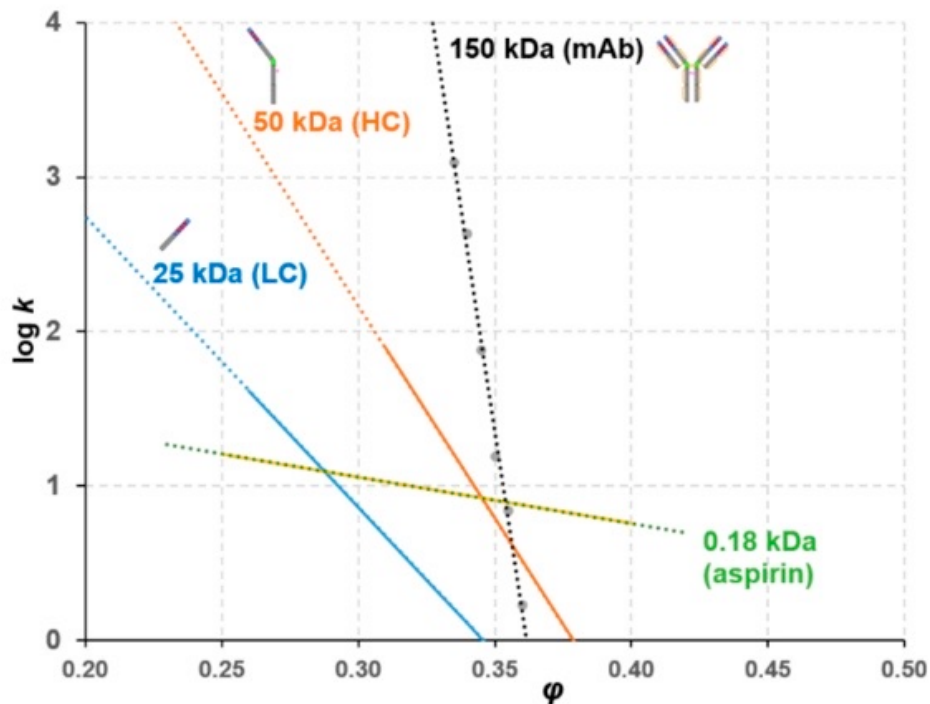
Extrapolation of bind-and-elute principles to other techniques



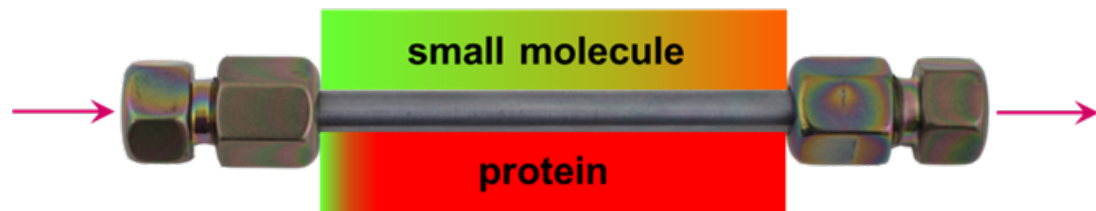
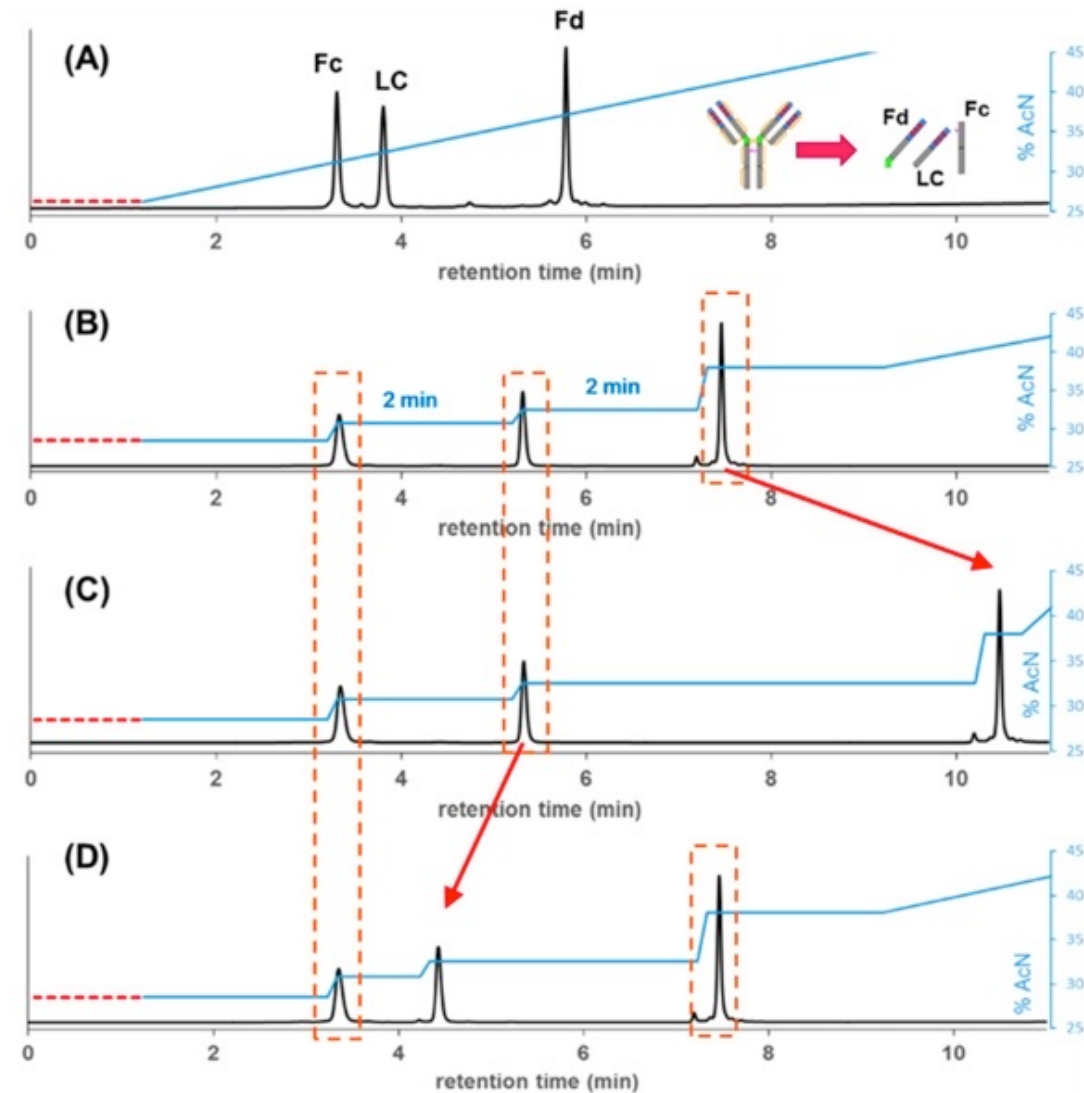
Are the current linear gradient profiles adequate?

Expending on the protein specific elution behavior

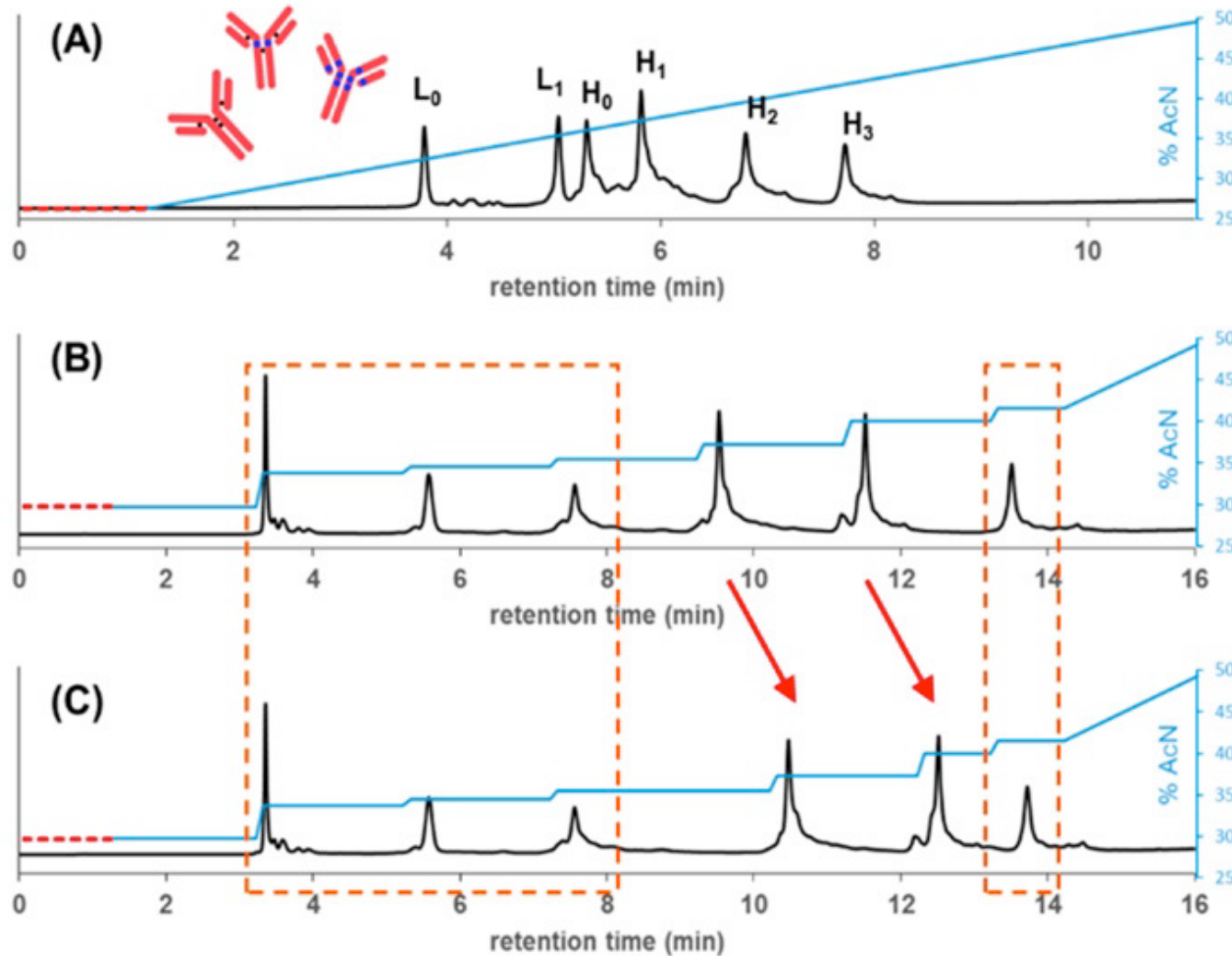
Using new multi-isocratic gradients for infinite selectivity



Retention via
Bind-Elute
principle



Application of multi-isocratic gradients to complex protein formats



Analysis of reduced Brentuximab Vedotin (Cysteine conjugated cytotoxic payload)

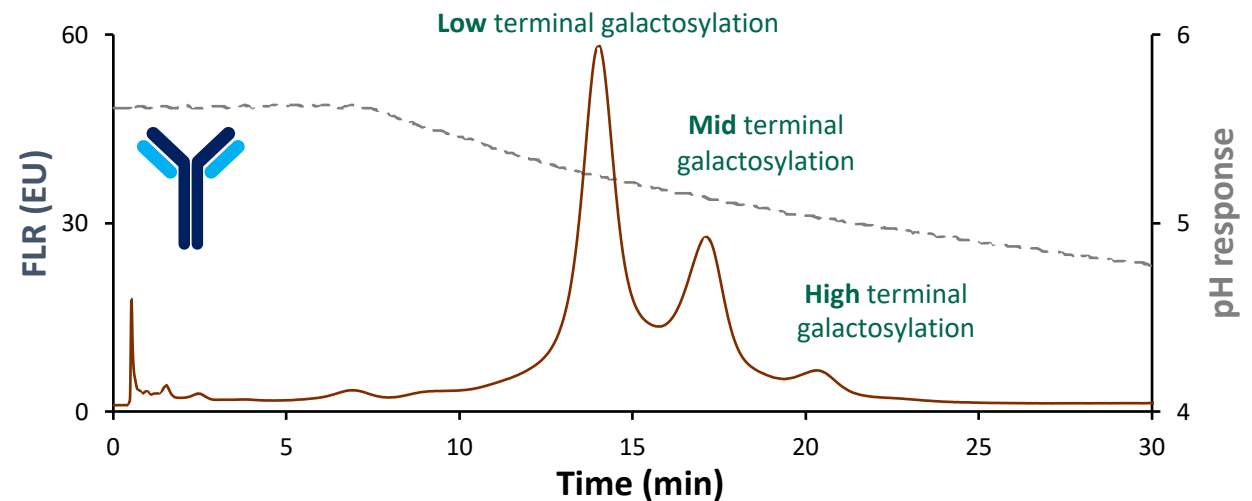
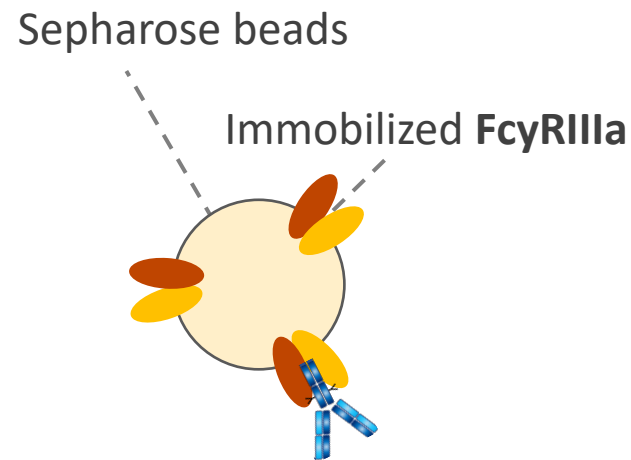
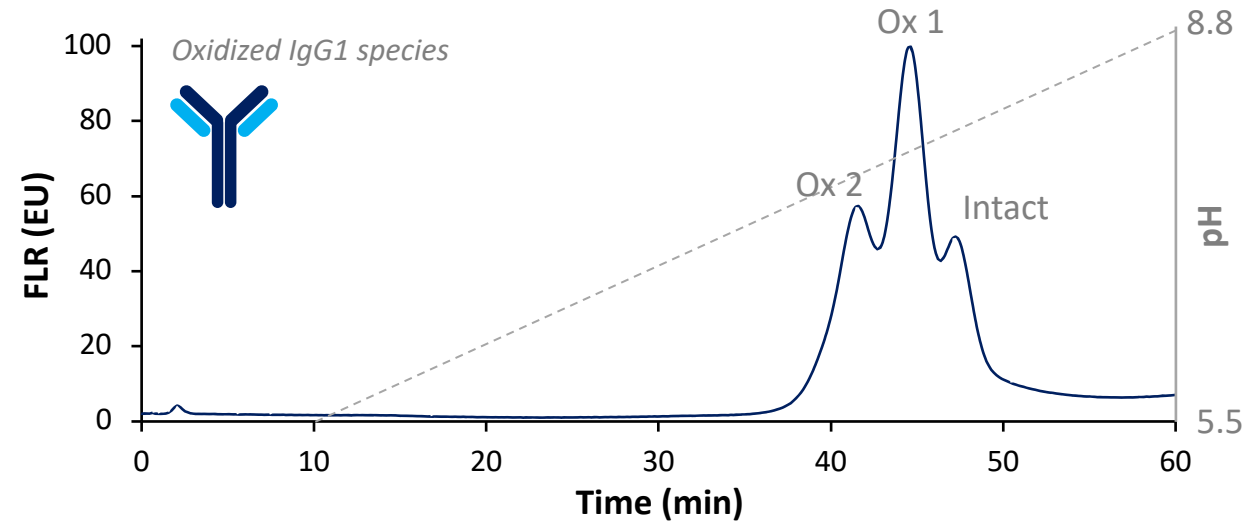
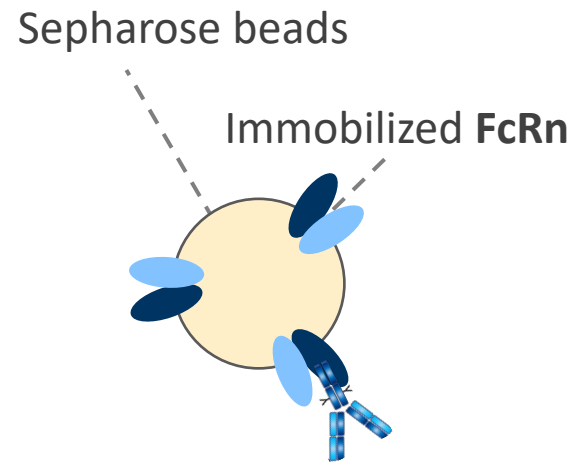
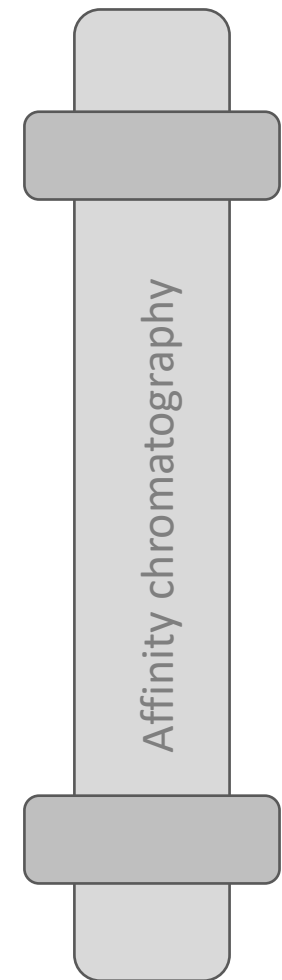
Application to:

- Bispecific antibodies
- Antibody-drug conjugates
- Full/Empty ratio for AAV's

Further developments:

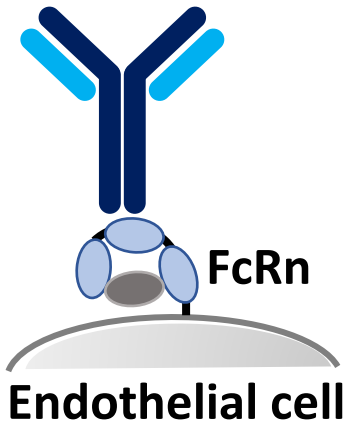
- Reduce analysis time
- Other chromatographic modes (HILIC/IEX)
- **Affinity chromatography (FcRn or FcγRIIIa)**

Conventional methods for FcRn and FcγRIIIa analysis



Affinity chromatography can help characterize the PK/PD effects of specific PTMs

Multi-isocratic segment methods for FcRn chromatography

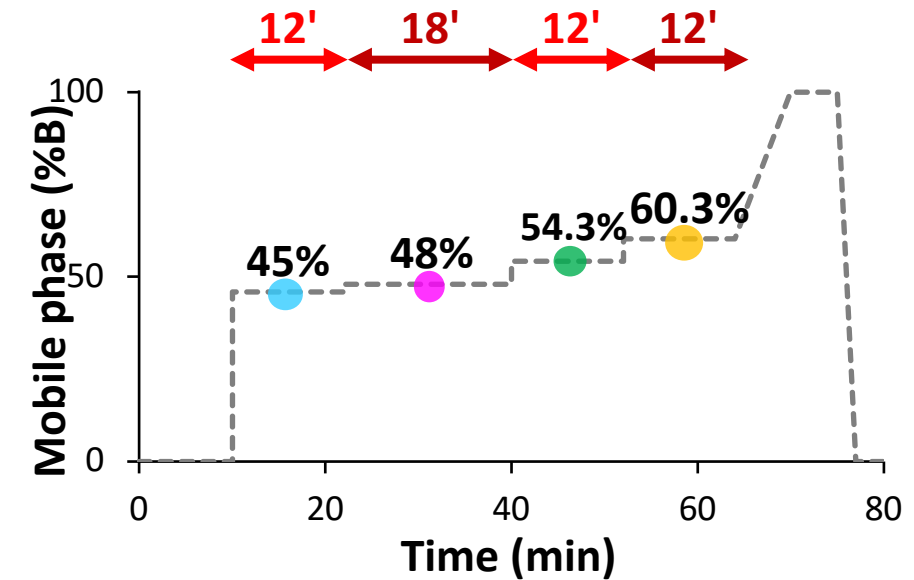
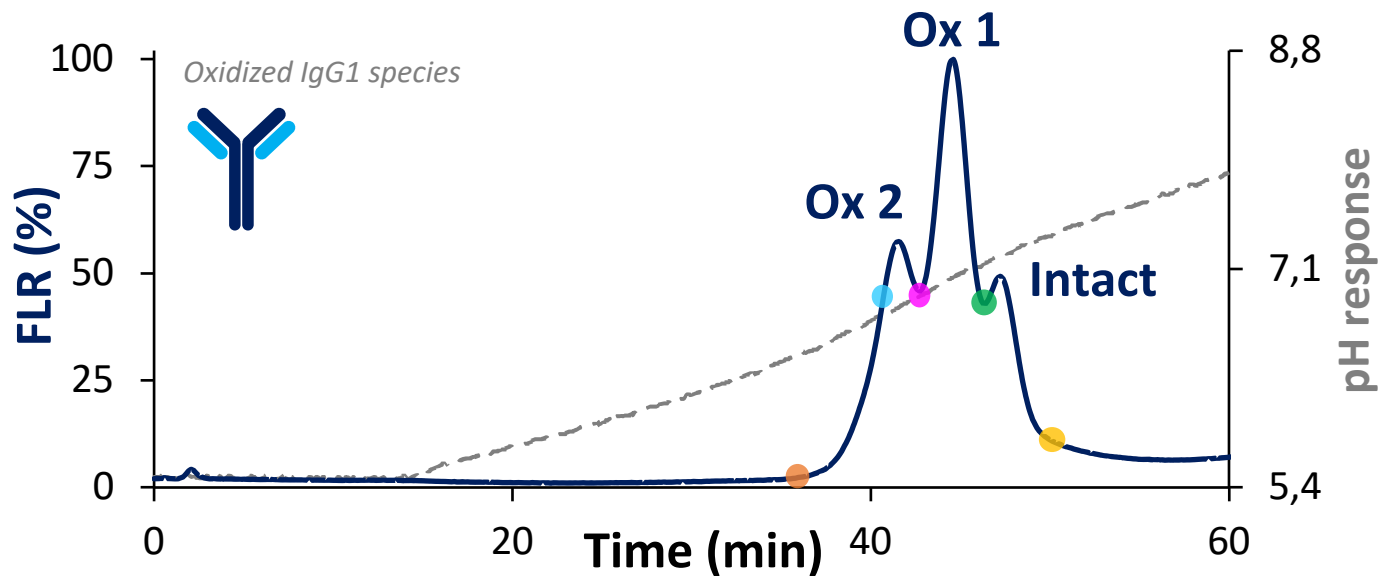


- On-Off behavior due to pH dependent receptor binding
- Therefore, Multi-isocratic gradient possible using a pH step-gradient

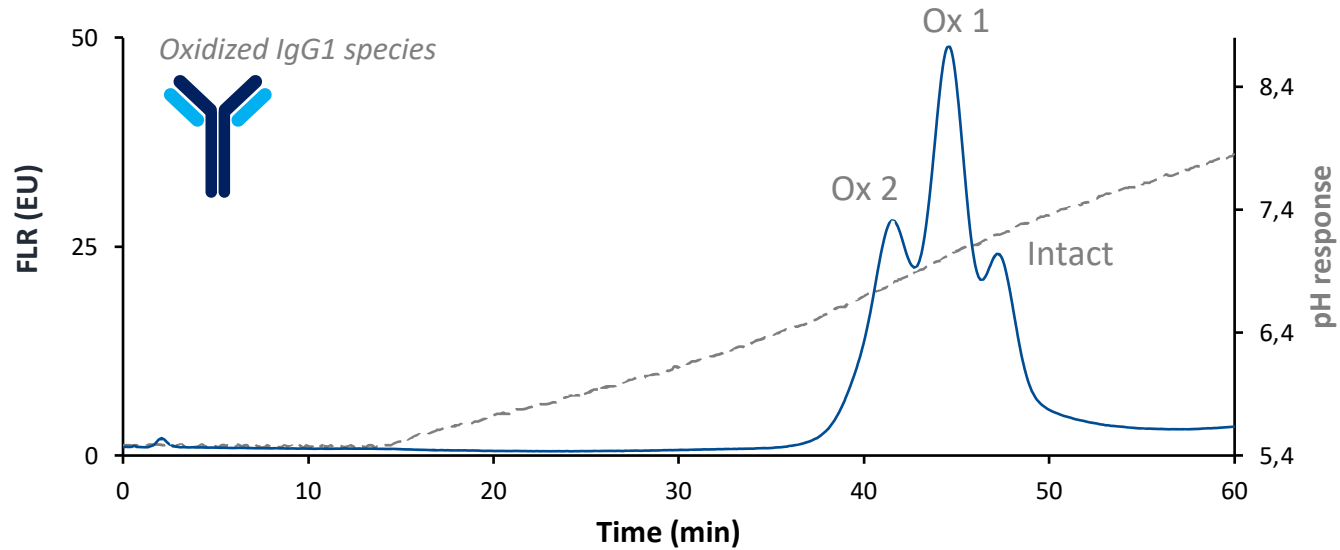
$$C_e = C_i + \frac{C_f - C_i}{t_g} \times (t_r - t_0 - t_D)$$

C_e = elution composition
 T_g = gradient time
 C_i = initial composition
 C_f = final composition

T_r = retention time
 T_0 = column dead time
 T_D = system dwell time

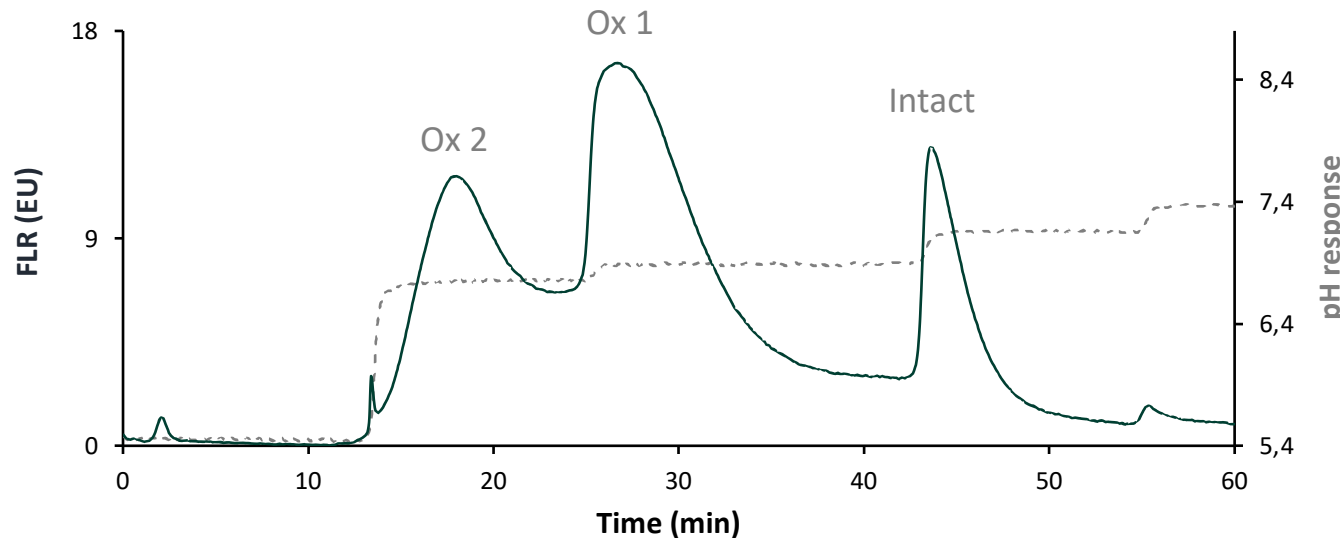


Multi isocratic segment methods in FcRn chromatography



Experimental conditions

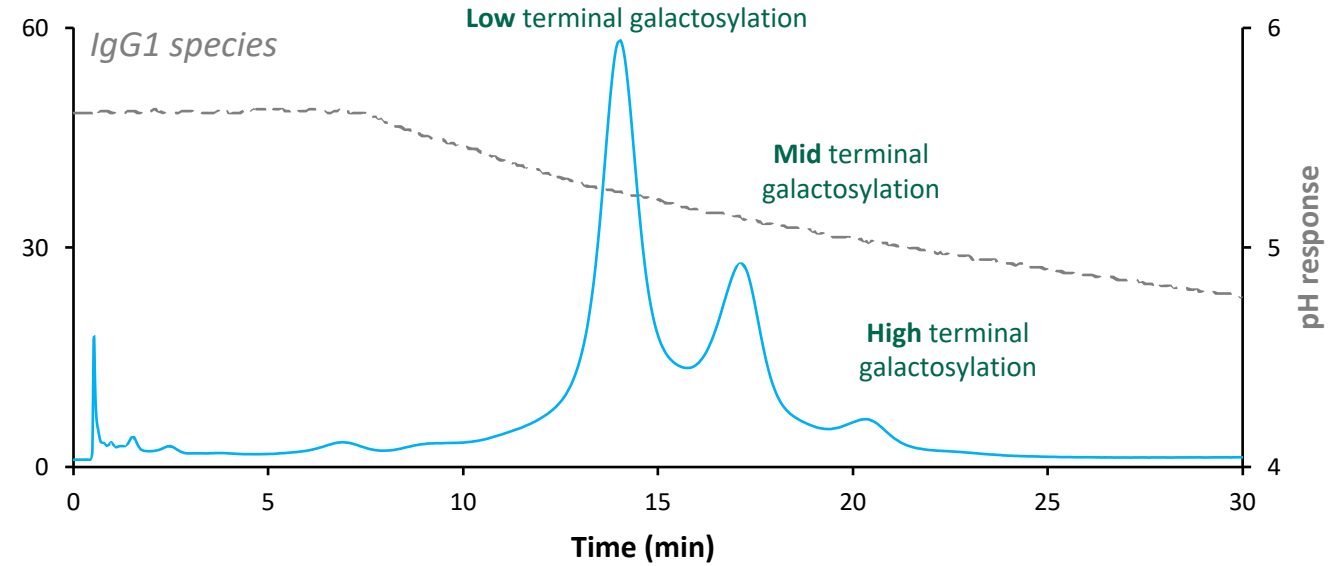
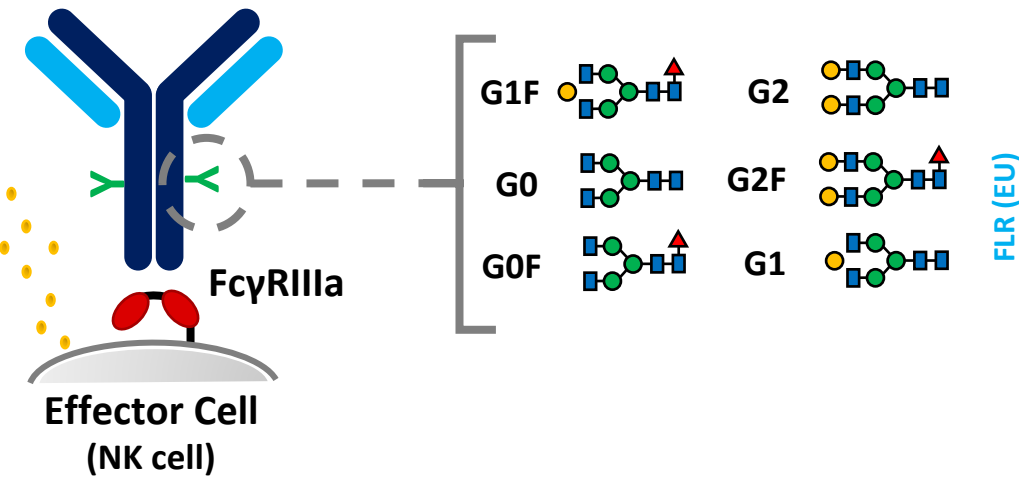
Roche FcRn affinity (5 x 50 mm)
A: 20 mM MES + 140 mM NaCl at pH 5.5
B: 20 mM HEPES + 140 mM NaCl at pH 8.8
Flow: 500 $\mu\text{L} \cdot \text{min}^{-1}$
FLD (280/340 nm)
Injection volume: 6 μL (0.5 $\text{mg} \cdot \text{mL}^{-1}$)



- Improvement of resolution between oxidized species due to the selection of the elution steps
- The space between peaks can be tuned by adjusting the length of the given **isocratic segment**

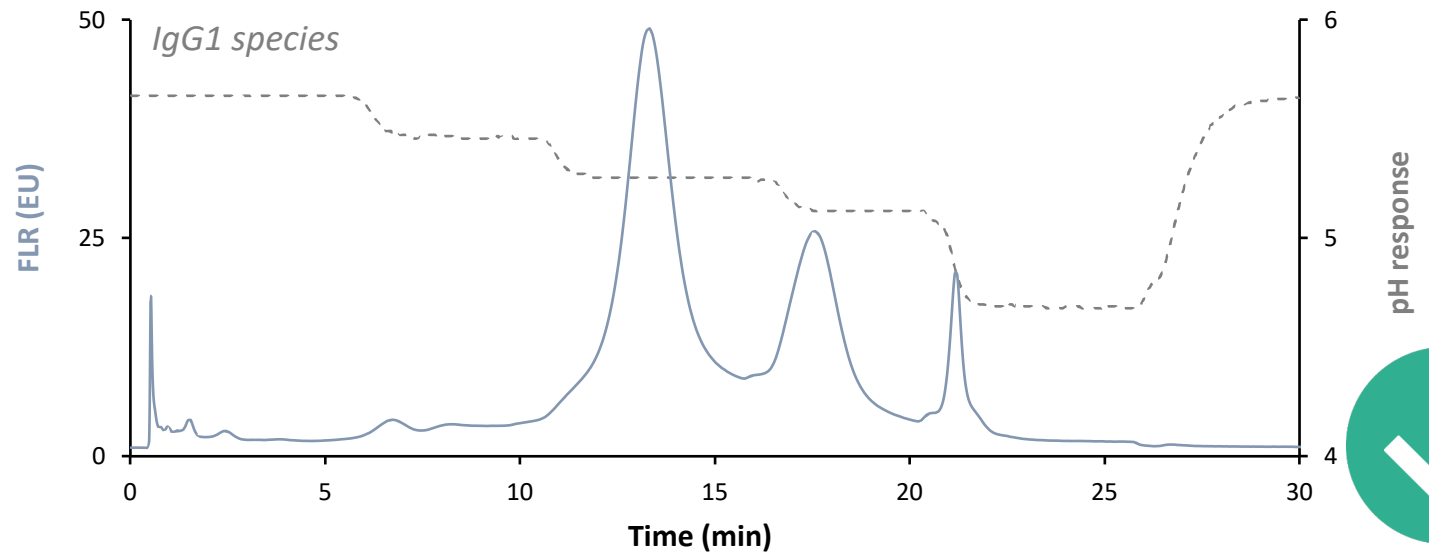


Multi isocratic segment methods in FcγRIIIa affinity chromatography



Experimental conditions

TSKgel FcR-IIIa-NPR (4.6 x 75 mm, 5 μm)
A: 50 mM sodium acetate + 150 mM NaCl (pH 6.5)
B: 50 mM citric acid + 150 mM NaCl (pH 4.0)
Flow: 1 mL.min⁻¹
FLD (280/340 nm)
Injection volume: 5 μL (1 mg.mL⁻¹)

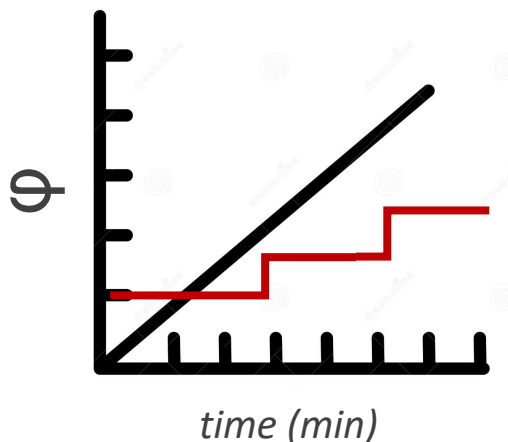


To conclude and to remember!



Ultra-short columns can greatly reduce the analysis time

- ✓ Protein analytes follow an on-off retention mechanism
- ✓ Multiple chromatographic modes can be transferred to short column formats
- ✓ Retention modelling software enables confident method development



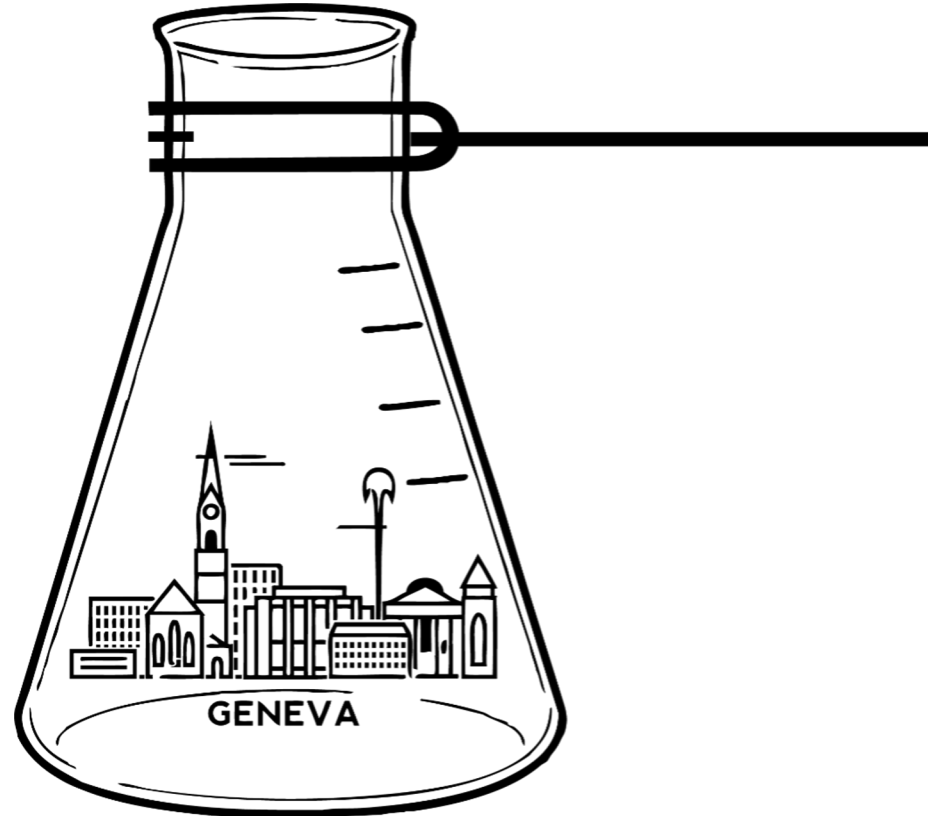
Multi-isocratic elution modes can be applied for affinity liquid chromatography

- ✓ The effect of PTMs on drug effector functions can be studied
- ✓ On-off retention mechanisms allow for improved resolution
- ✓ Potential combination of functional and physiochemical characterization setups

ACKNOWLEDGEMENTS



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