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

Antigen binding domain
- Targeting potential of mAbs
- Blocking of receptors
- Neutralization of antigens

Immune mediated effector functions
- Cell-killing potential
- ADCC via FcyR binding
- CDC via C1q binding

Half life extension
- FcRn- receptor mediated recycling

Antibodies are ~ 800 times larger than paracetamol!

ADCC = Antibody-dependent cellular cytotoxicity, CDC = Complement-dependent cytotoxicity
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
- Asn

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)
- Columns: 50 – 150 x 2.1 mm
- Analysis time: 5 – 20 minutes

II. Ion-Exchange (IEX)
- Released glycan analysis on standard pore stationary phases

III. Hydrophilic Interaction (HILIC)
- Columns: 150 x 2.1 mm
- Analysis time: 30 - 40 minutes

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, $k = 100$ (34% ACN) and $k = 1$ (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

After traveling only 5 mm, the mAb retention drops drastically. 

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

Conventional column → Ultra-short columns

Ultra-short columns

0 cm 1 cm 2 cm 3 cm 4 cm 5 cm

Aspirin

K

φ

0 cm 1 cm 2 cm 3 cm 4 cm 5 cm

0 2 cm 3 cm 4 cm 5 cm

0 cm 1 cm 2 cm 3 cm 4 cm 5 cm

0 2 cm 3 cm 4 cm 5 cm

K

φ

0 cm 1 cm 2 cm 3 cm 4 cm 5 cm

0 2 cm 3 cm 4 cm 5 cm

K

φ

0 cm 1 cm 2 cm 3 cm 4 cm 5 cm

0 2 cm 3 cm 4 cm 5 cm

K

φ
Application to RPLC analysis of anti-COVID therapeutics

Sample preparation

Imdevimab
75 °C, 26–36 %B

Experimental parameters

Bioresolve RP mAb Polyphenyl
(50 x 2.1mm, 2.7µm, 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

Source: Duivelshof et al. (2022) JPBA
From optimized conditions, virtually transferring to ultra-short columns

Important parameters when Transferring methods:
- Switch to low dwell and extra column volumes
- Change to ultrashort column parameters
- Change flow rate and gradient

Source: Duivelshof et al. (2022) JPBA

Software: Drylab, Chromsword, etc.
Extrapolation of bind-and–elute principles to other techniques

Are the current linear gradient profiles adequate?
Expanding 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

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 FcyRIIa)

Analysis of reduced Brentuximab Vedotin (Cysteine conjugated cytotoxic payload)

Conventional methods for FcRn and FcyRIIIa analysis

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

Source: Bouvarel et al. (2022) J. Chrom. A.
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

Oxidized IgG1 species

Source: Bouvarel et al. (2022) J. Chrom. A.
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 µL.min\(^{-1}\)
FLD (280/340 nm)
Injection volume: 6 µL (0.5 mg.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

Source: Bouvarel et al. (2022) J. Chrom. A.
Multi isocratic segment methods in FcyRIIIa 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)

Source: Bouvarel et al. (2022) J. Chrom. A.
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
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