De la chimie à la biologie: les nouvelles frontières de l'analyse des médicaments de dernière génération

26ème Journée Scientifique du cCCTA « Biologie-Chimie, l'entre deux mondes…»

14 – 15 septembre, Villars-sur-Ollon, Suisse

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Where are we in terms of new modalities?

MATURE
Clinically proven and in wide use

ADC
Bispecific antibodies
Gene therapy
RNAi and oligos
mRNA (prophylactic vaccine)
Chimeric antigen receptor (CAR)
T-cell therapy

EMERGING
Still in preclinical or early clinical studies

High ←←←←← TECHNOLOGICAL MATURITY →→→→→ Low

Small molecules
Recombinant proteins
Monoclonal antibodies

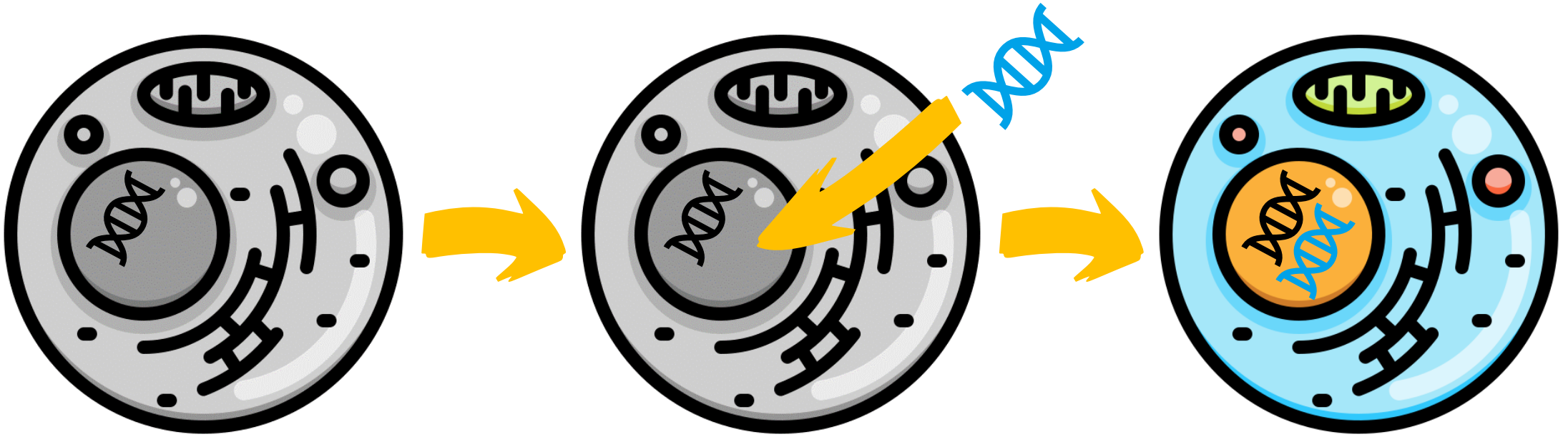
ADVANCING
Passed the initial proof of concept and are through late-stage clinical studies or already commercialized

mRNA (therapeutic and vaccines)
Stem cell therapy
Tumor-infiltrating lymphocytes (TILs), CAR-NK, CAR-M, or T cell receptor T (TCR-T) cell therapy
Proteolysis-targeting chimeras (PROTACs)
Microbiome
Oncolytic virus
Gene editing

https://www.bcg.com/publications/2023/benefits-and-risks-of-new-drug-modalities

What is gene therapy?

Therapy that targets the genetic root cause of a specific disease



Cell has defective gene

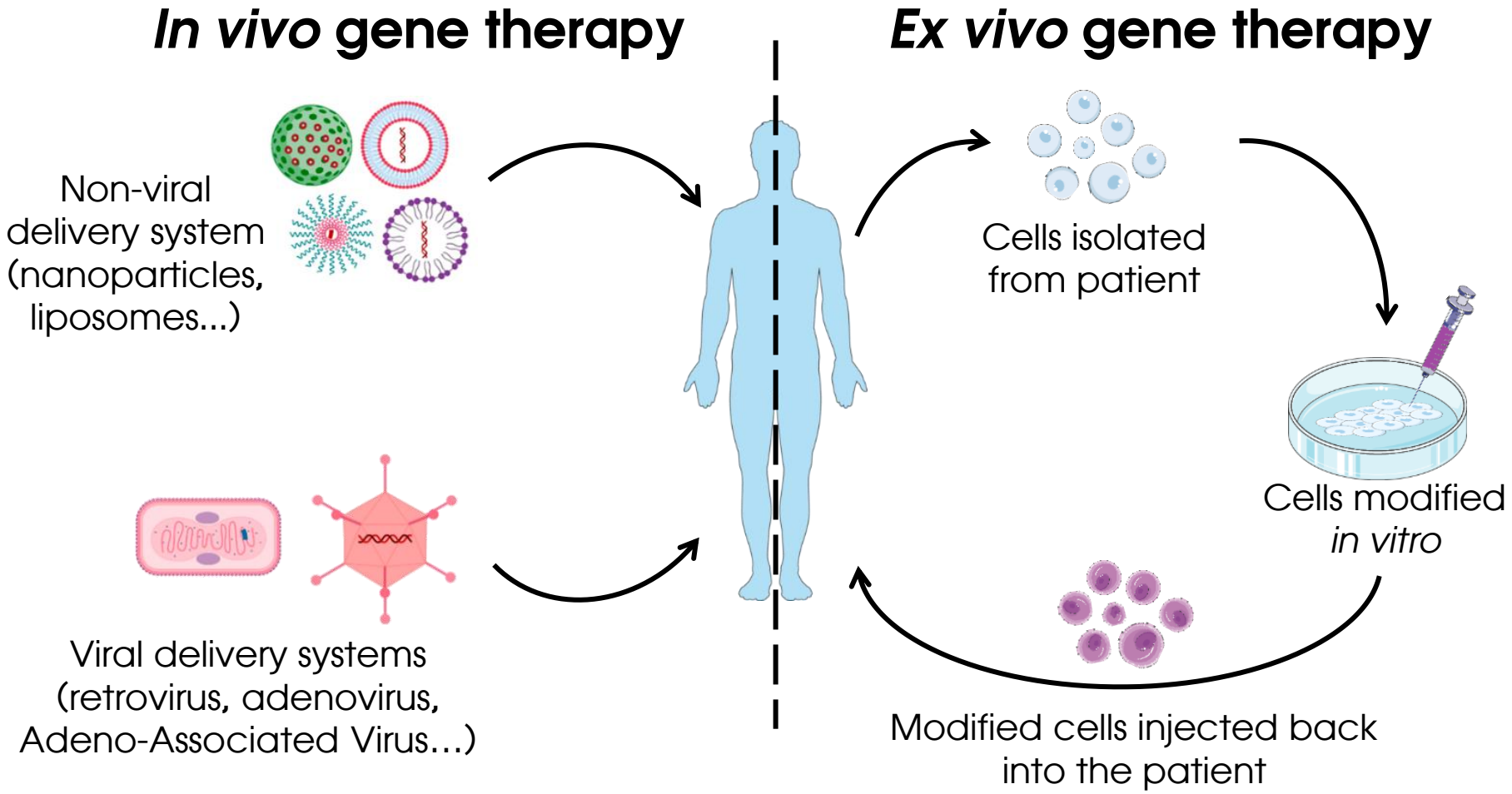
Healthy gene is introduced

Cell function is restored

A working copy of the defective gene is delivered as therapeutic drug

How to deliver a therapeutic gene?

Two different strategies are possible for delivering a therapeutic gene



Timeline of the FDA-approved gene therapies

Three AAV-based therapies currently approved by the FDA

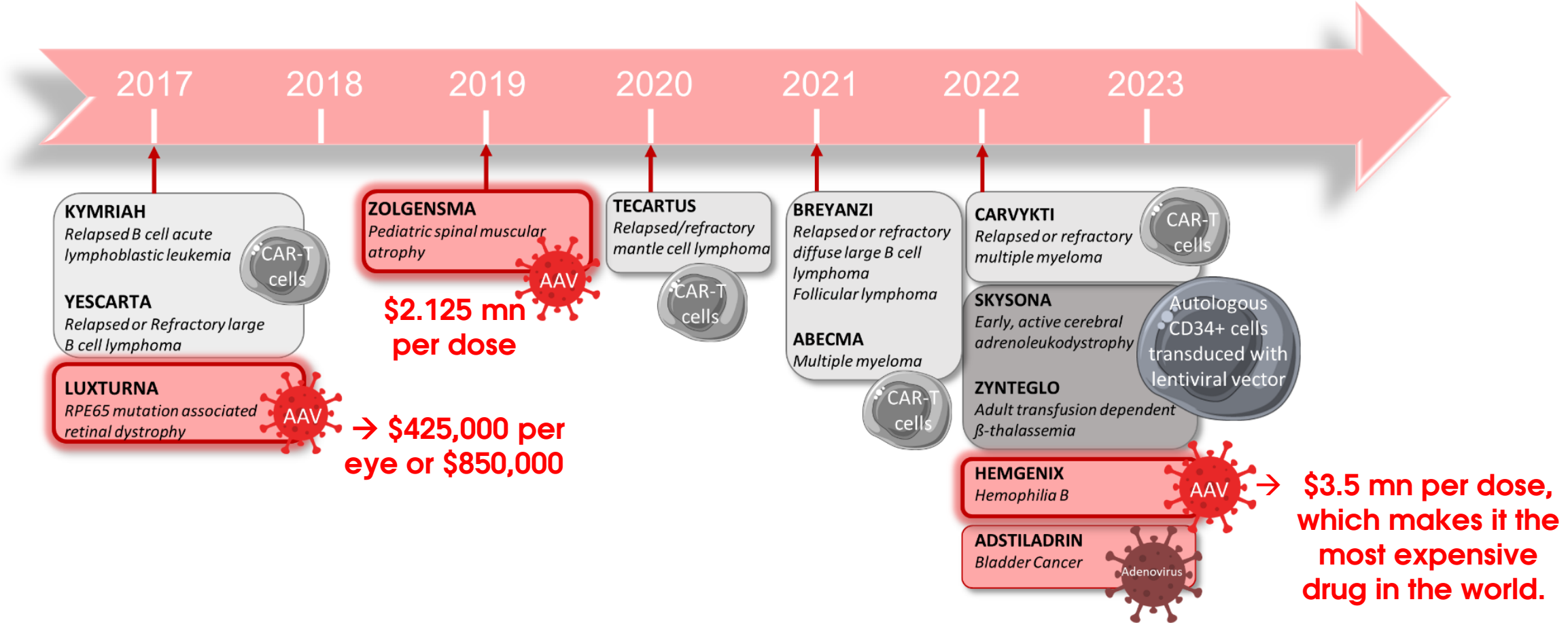
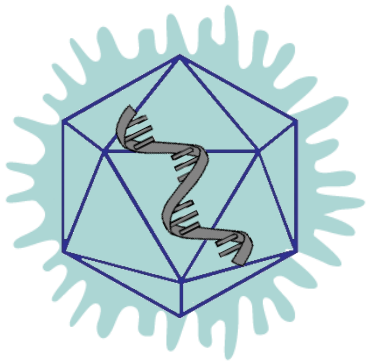


Figure ref = Fekete et al. TrAC 2023, 117088

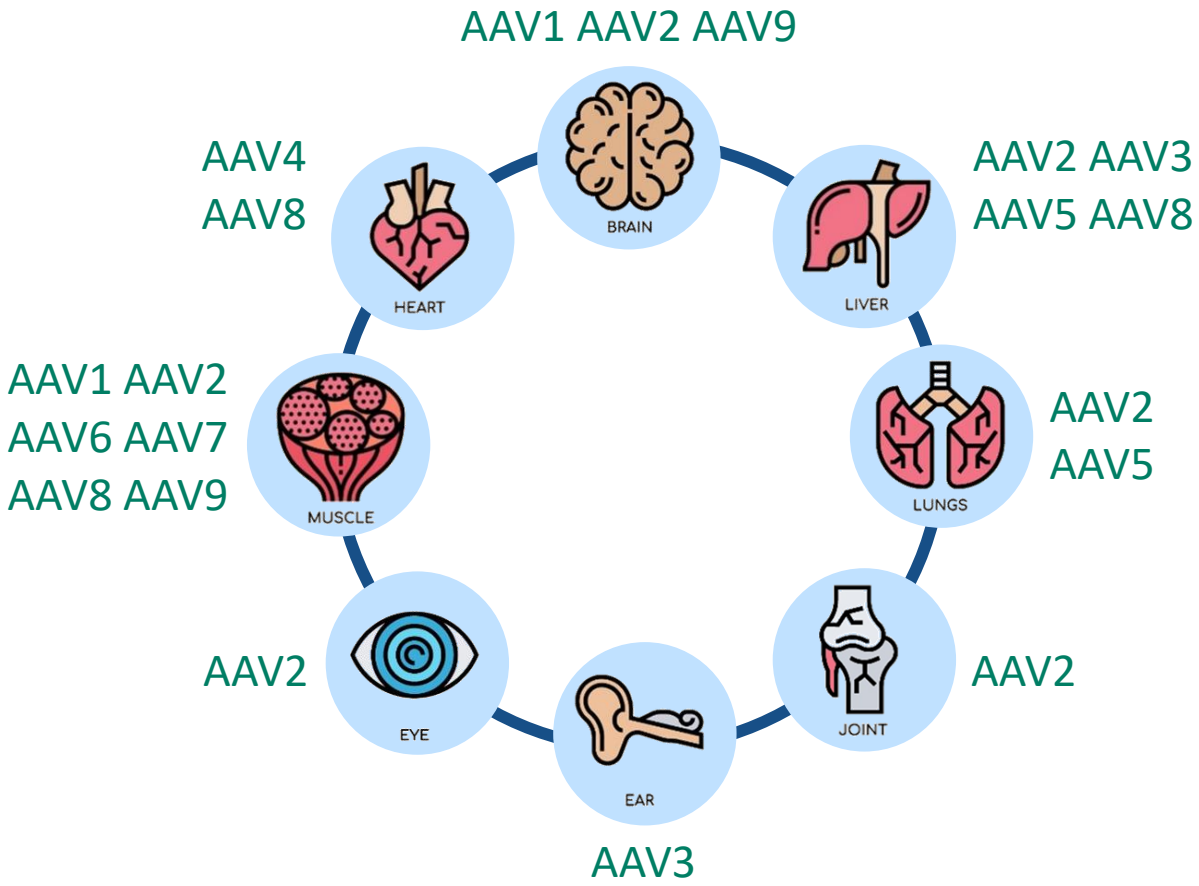
* https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products

Adeno-Associated Virus (AAV) as vectors for gene delivery

AAV are a safe and attractive vectors for gene delivery



- Non-pathogenic single stranded DNA virus
- Small viral genome (ss DNA < 4.8 kb)
- Icosahedric capsid consisting of ~60 viral proteins (VP1, VP2, VP3)
- Diameter = 22 nm
  Mass = 3700 kDa
- 12 different serotypes



AAVs serotype have specific target tissue tropism

Analytical methods to be stablished for AAV-based gene therapy

Parameter	Method
Full/Empty Capsid	AUC (gold standard), TEM (gold standard), IEX, SEC-MALS, CE-SDS/cIEF
Aggregation and Fragmentation	SEC, SEC-MALS, DLS
Capsid Integrity (VP1-3 Ratio)	RPLC, CE-SDS/cIEF
Capsid Identity (VP1-3 Ratio), PTM	LC-MS
Particle Concentration (viral titer)	SEC-MALS, ELISA
Genomic titer, genomic identity	qPCR/ddPCR
Infectivity, potency, mode of action	Cell based assays

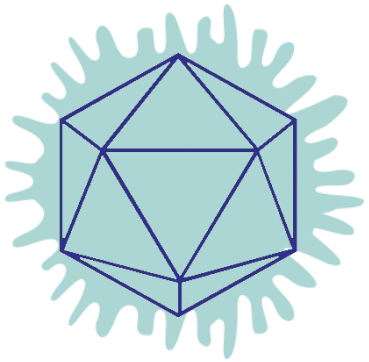
AUC = Analytical Ultra Centrifugation; TEM = Transmission Electron Microscopy; IEX = Ion Exchange Chromatography, SEC = Size Exclusion Chromatography; MALS = Multi-angle Light Scattering; DLS = Dynamic Light Scattering; ELISA = Enzyme-Linked Immunosorbent Assay; RPLC = Reversed-Phase Liquid Chromatography; CE-SDS = Capillary electrophoresis sodium dodecyl sulfate; cIEF = capillary IsoElectric Focusing; q/ddPCR = quantitative/droplet digital Polymerase Chain Reaction

IEX method to quantify Full vs Empty capsids

Empty rAAV capsids represent a product-related impurity that need to be monitored and quantified as represent a safety concern.

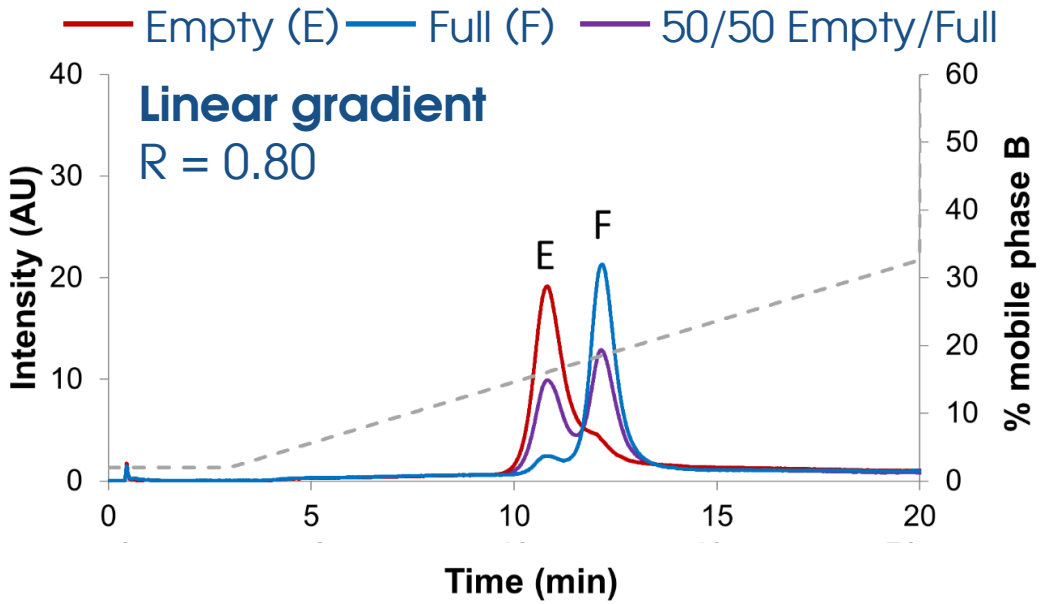
Generic salt-mediated AEX gradient conditions for rAAV Empty and Full capsids separation

Product related impurity



EMPTY

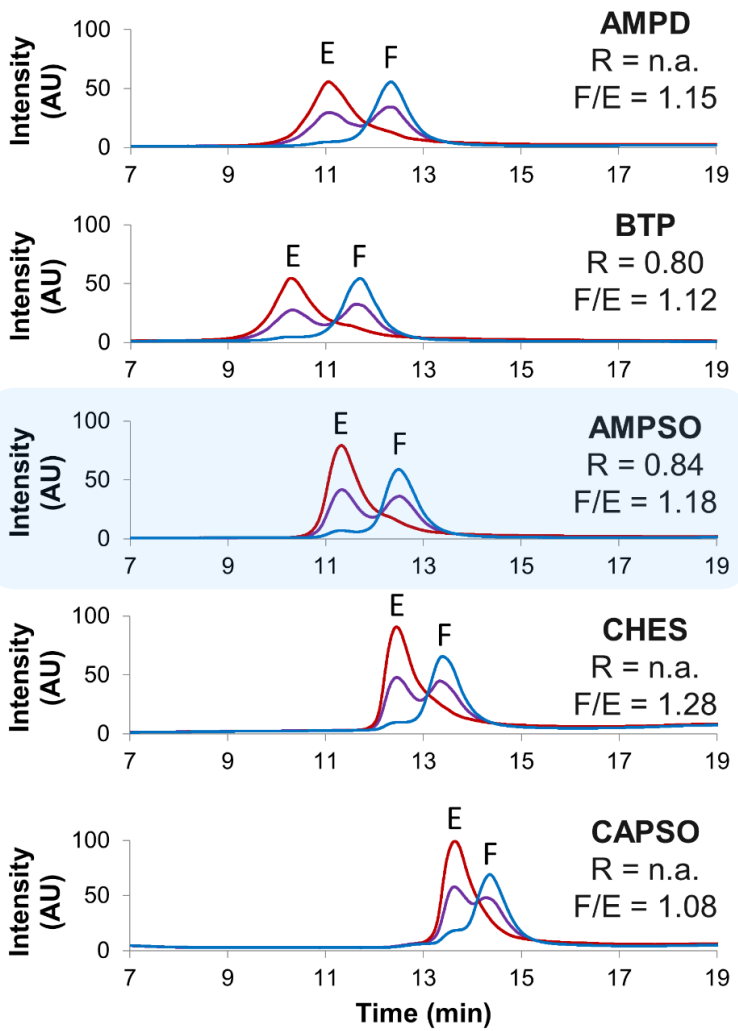
→ Reduce effective concentration
→ Compete for binding sites
→ Increase immunogenicity
→ Reduce efficacy

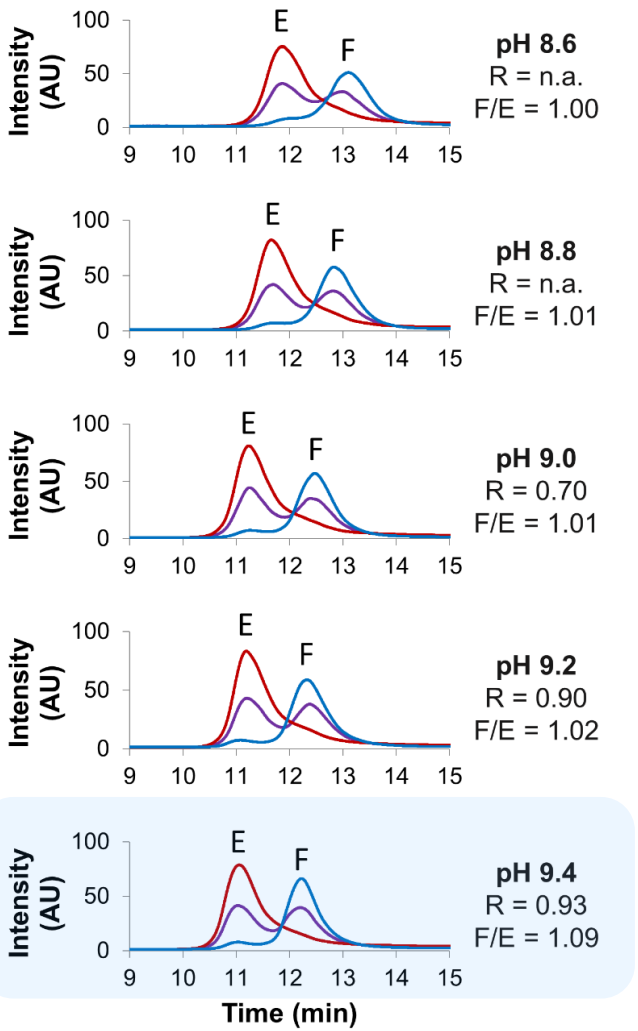


Issue = Empty and Full rAAV capsids have the same size and minor p*I* difference (0.4 pH) so poor chromatographic resolution is generally obtained.

Gradient conditions = linear gradient of BTP buffer pH 9.0 in combination with NaCl as eluent salt. FLD detection = λ_{ex} at 280 nm and λ_{em} at 350 nm. Injection volume = 20 µL of 1.00 E+12 vp/mL samples diluted in water. Column = Thermo ProPac SAX-10 50 x 4 mm, 10 µm (nonporous PEEK column hardware)

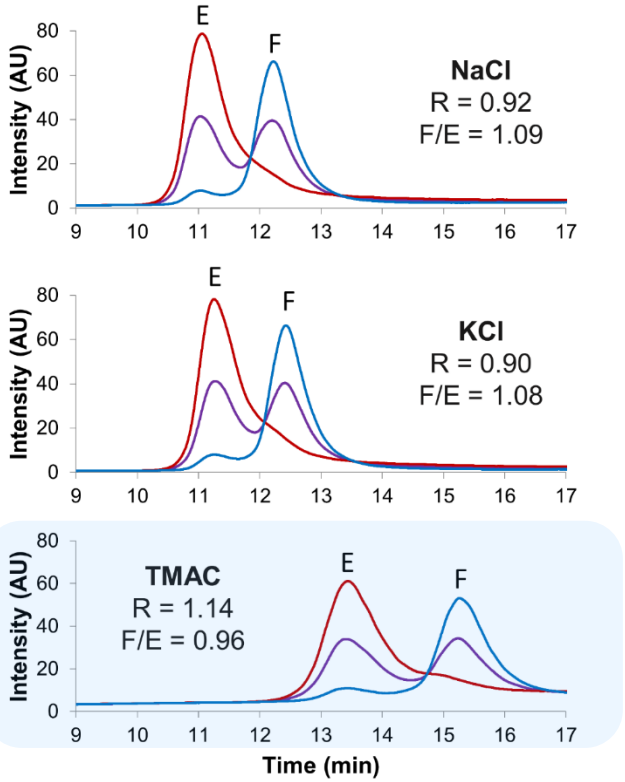
Buffer scouting plus pH and salt screening





Empty (E) — Full (F) — 50/50 Empty/Full

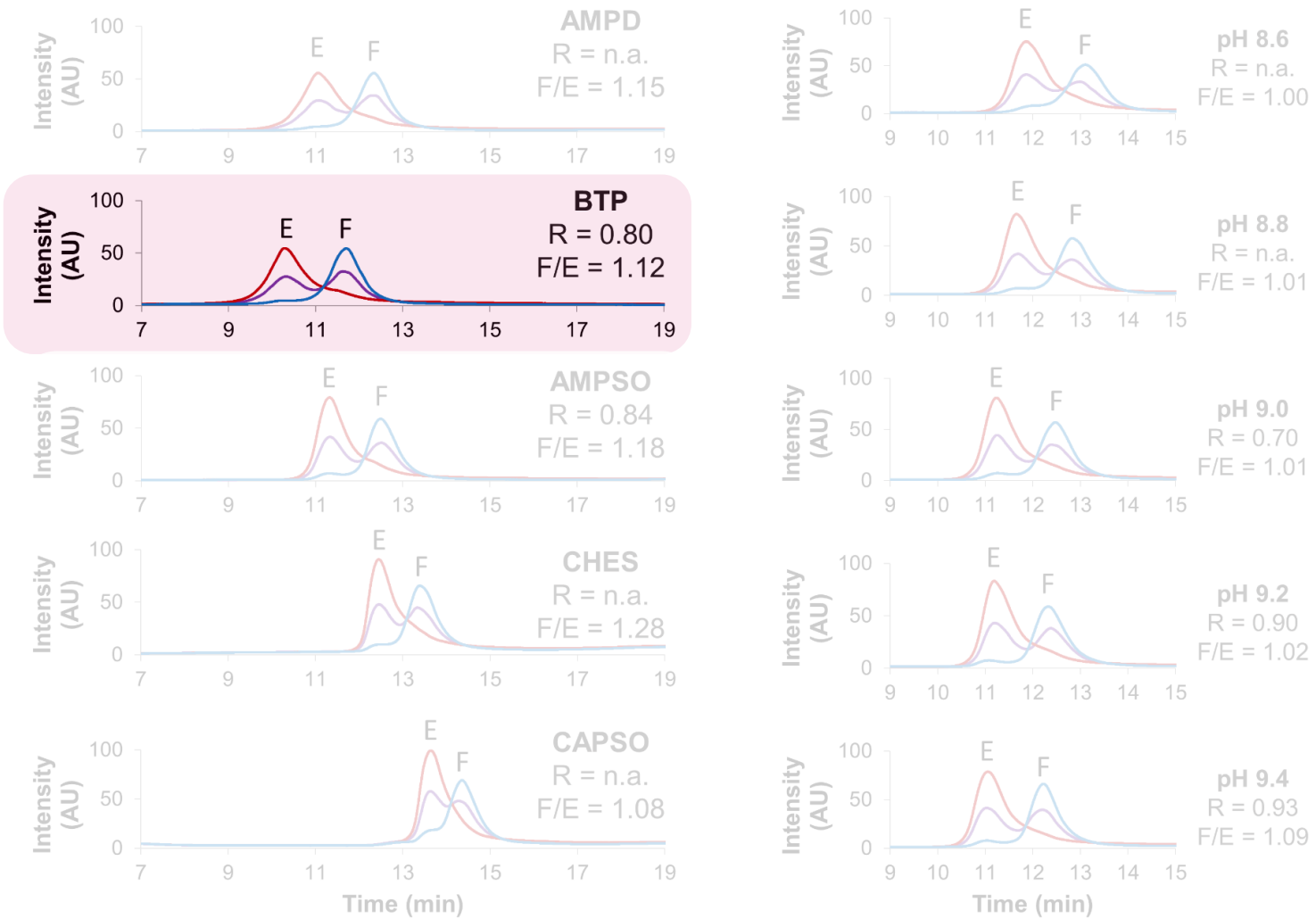
Higher resolution obtained when using tetramethylammonium chloride (TMAC) as eluent salt.



→ Gradient conditions: linear gradient of AMPSO buffer pH 9.4 in combination with different eluent salts.

Aebischer et al. IJMS 2022, 23, 12332

Linear gradient optimization is not enough



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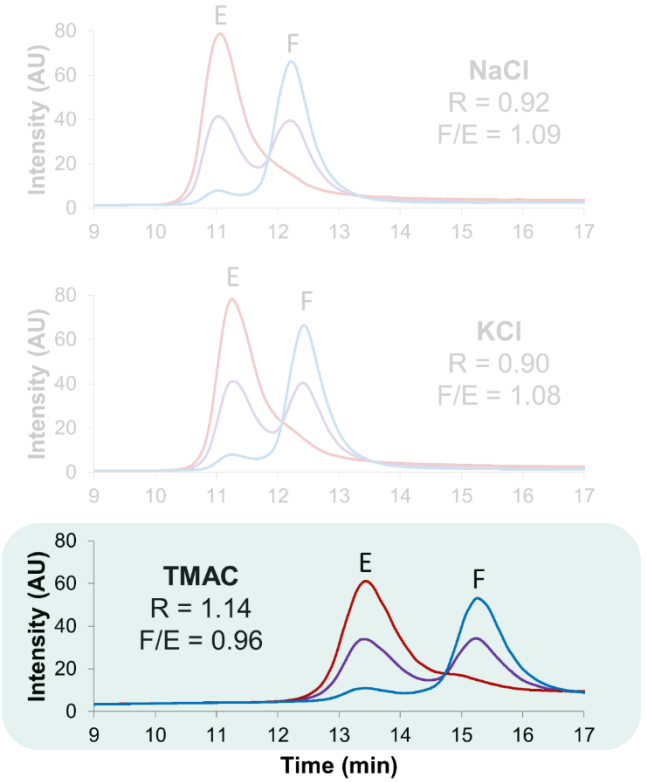


→ Gradient conditions: linear gradient of AMPSO buffer pH 9.4 in combination with different eluent salts.

Aebischer et al. IJMS 2022, 23, 12332

ON/OFF mechanism of large molecules (proteins)

AAV capsids are made of proteins, do they follow an "on/off" retention behavior?

LSS model describes the relationship between solute retention (***k***) and mobile phase composition (***φ***)

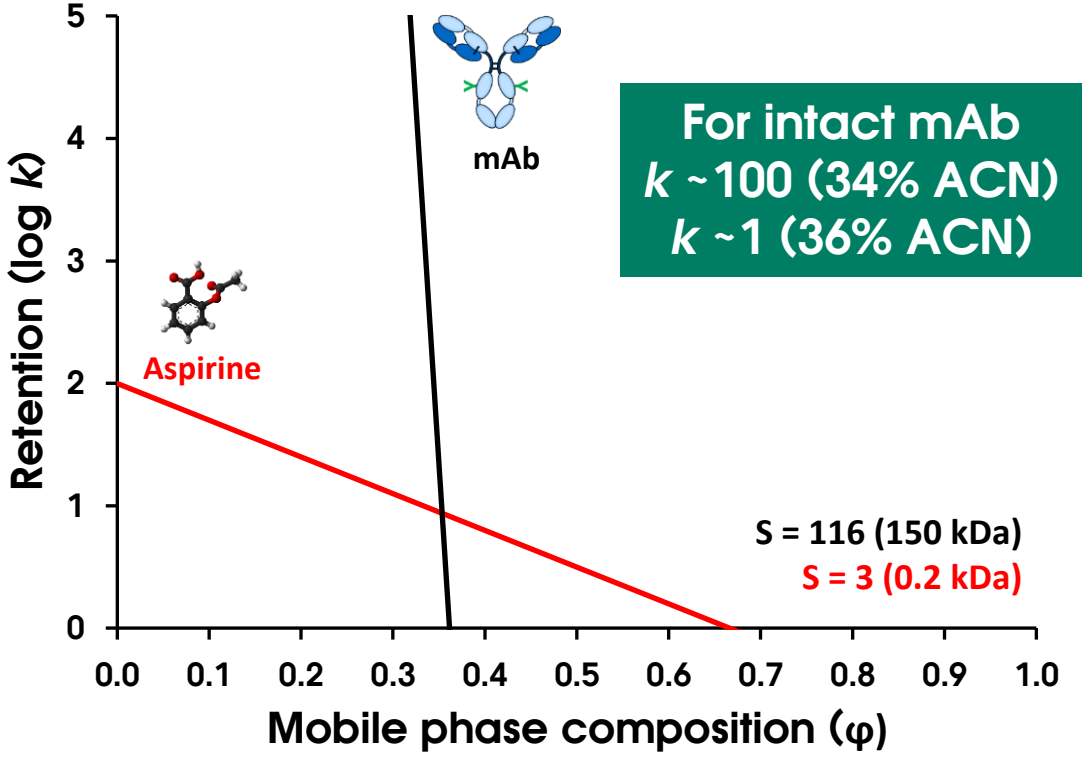
$$\log k = \log k_0 - S \times \varphi$$

k = retention factor
φ = volume fraction of mobile phase "B" (stronger eluent)
S = constant for a given solute
(*describes how sensitive is the retention to φ*)
k_0 = (extrapolated) value of *k* for *φ* = 0

The slope of these curves drastically increases with the size of the molecule

Large molecules follow an "**ON-OFF**" or "**Bind-and-elute**" retention mechanism

Linear Solvent Strength (LSS)



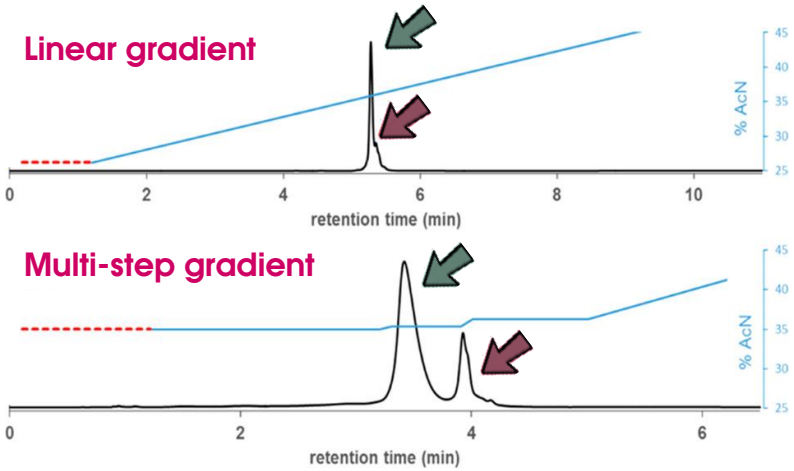
Fekete et al. *Analytical Chemistry* 2019, 91, 12954-12961

ON/OFF mechanism of large molecules (proteins)

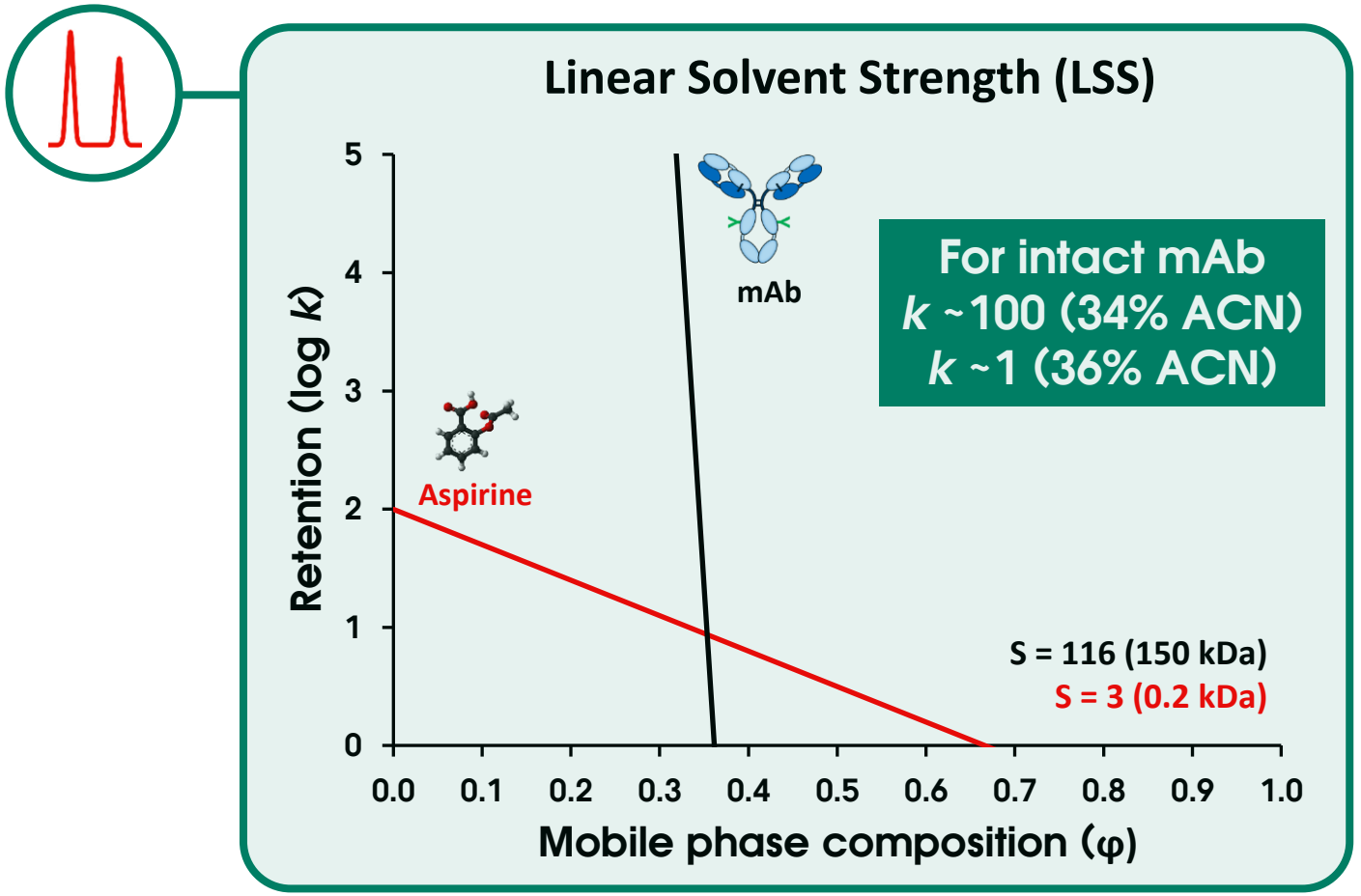
AAV capsids are made of proteins, do they follow an "on/off" retention behavior?

Large molecules (proteins) are not eluted until exposed to a given mobile phase composition!

Selectivity can be arbitrarily changed between peak pairs by alternating several gradients and isocratic steps.



Fekete et al. *Analytical Chemistry* 2021, 93, 1277-1284



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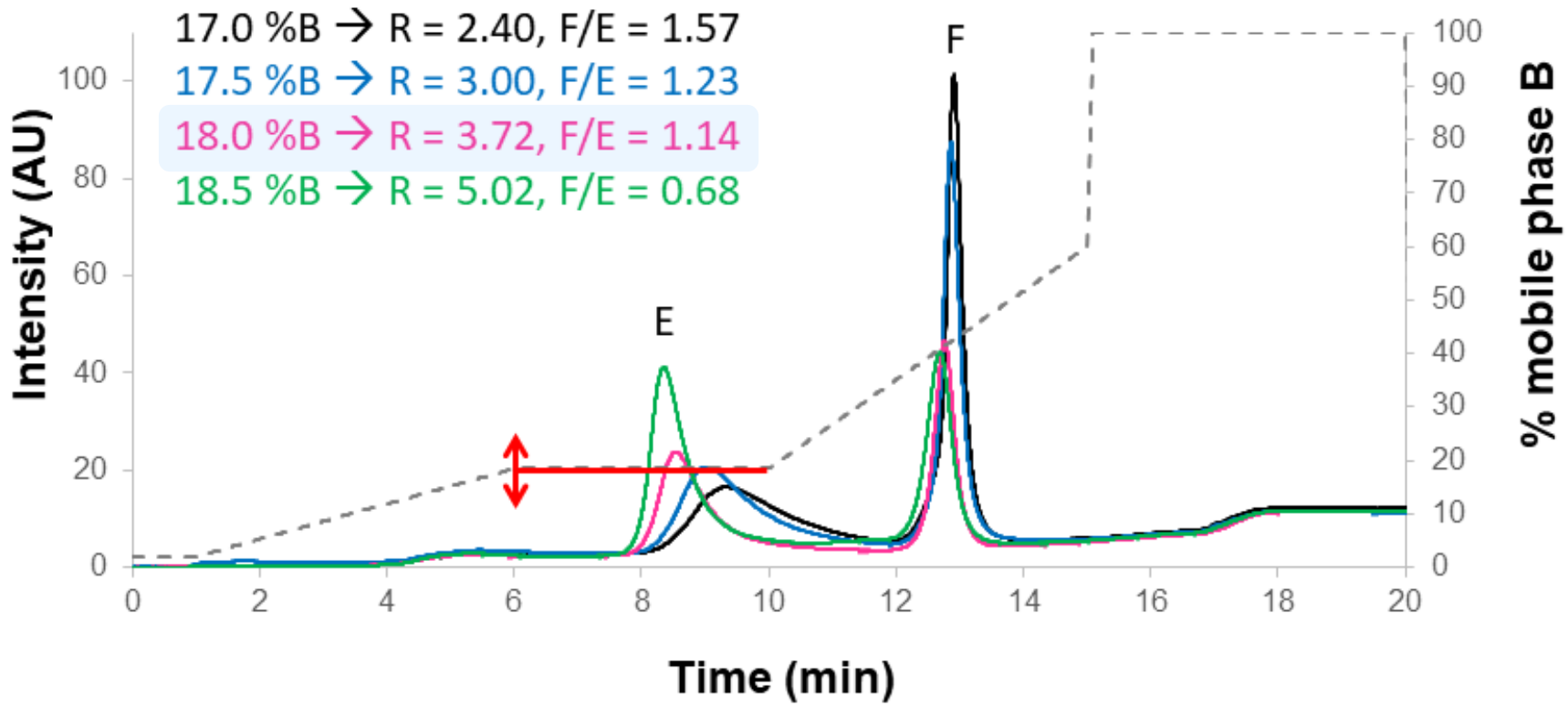
Improved selectivity by using multi-isocratic elution

AAV capsids are made of proteins, do they follow an "on/off" retention behavior?

Optimal gradient conditions modeled by DryLab → Isocratic step at 17.5 %B.



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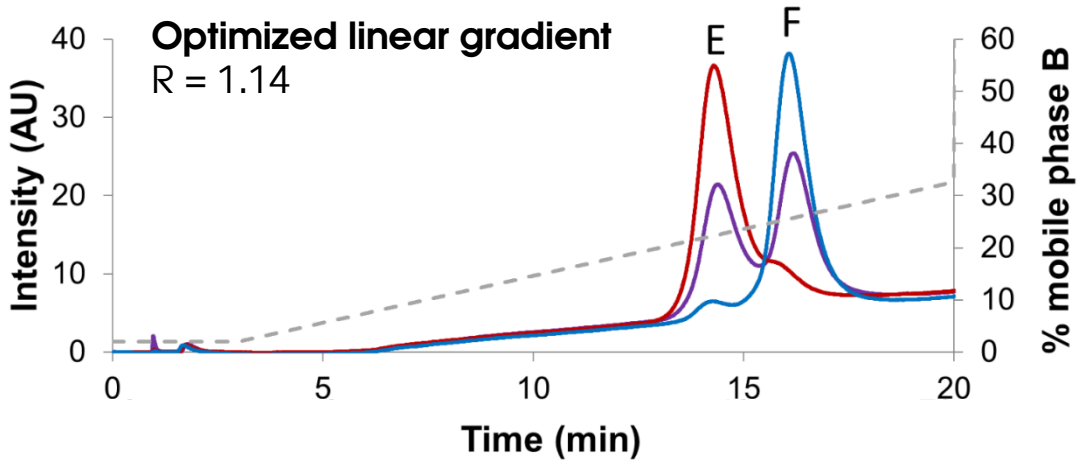

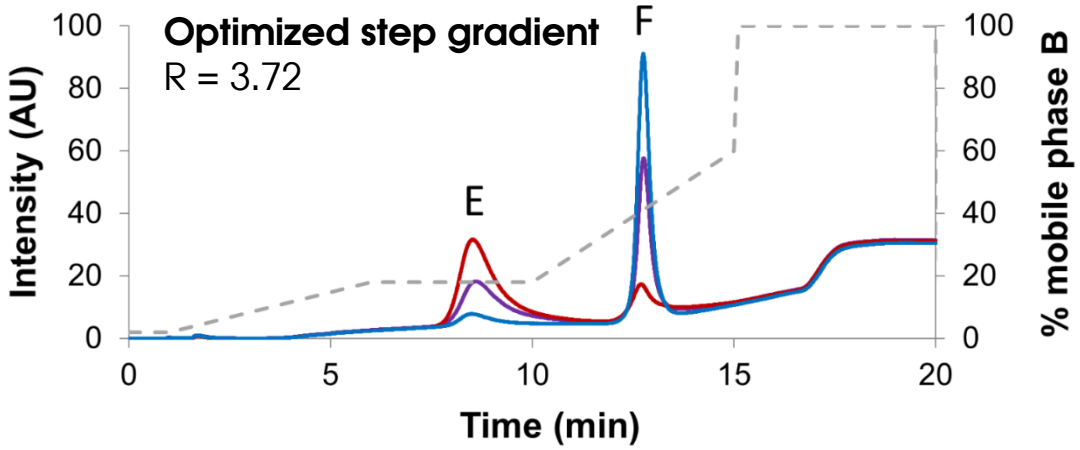

Remarkable improved resolution obtained when applying the step gradient method!

Empty (E) — Full (F) — 50/50 Empty/Full

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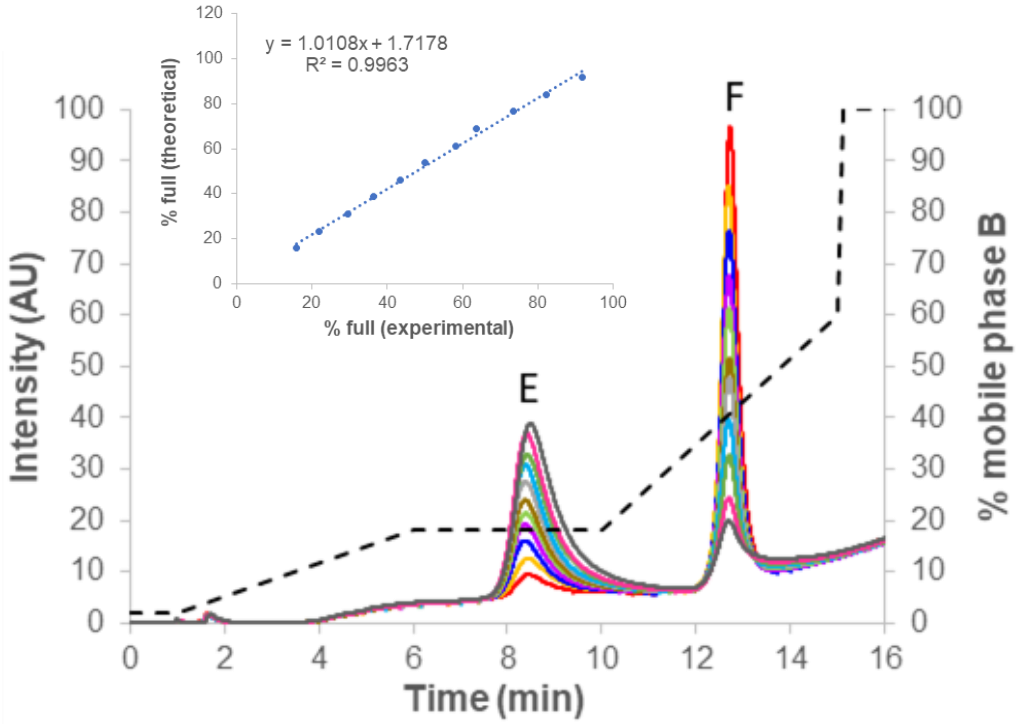
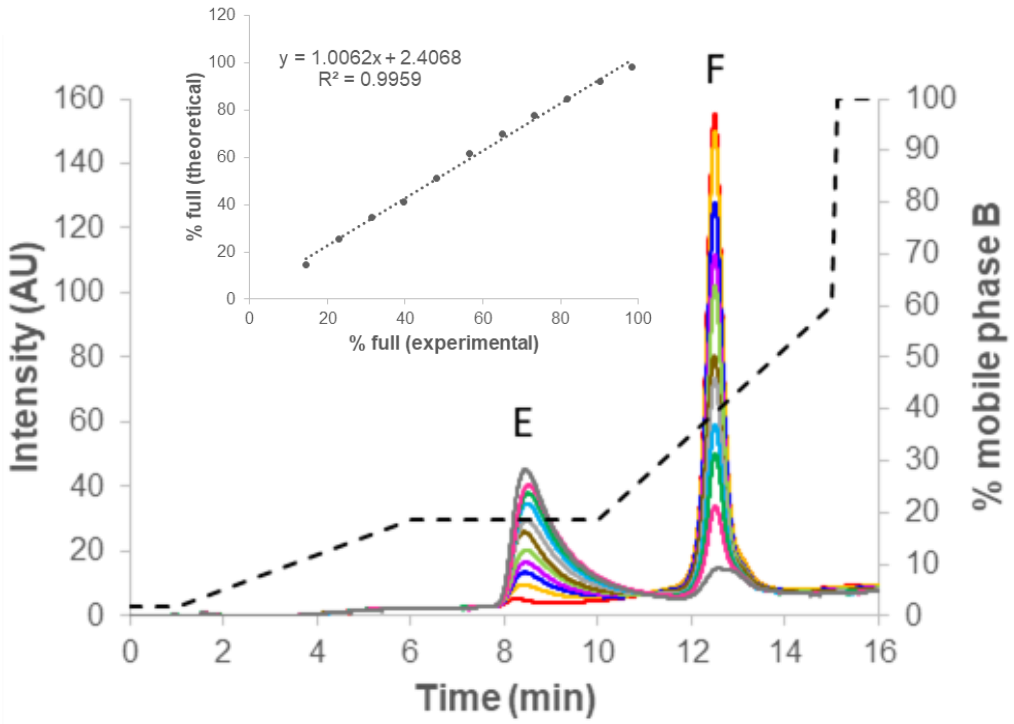
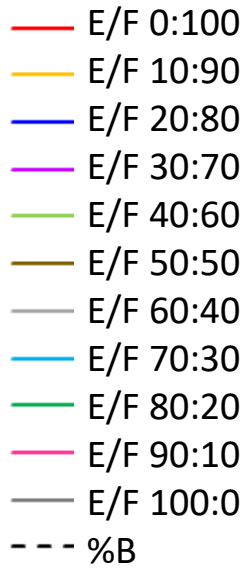
Method validation by Full/Empty quantification

Samples from Virovek



Samples from Sirion Biotech



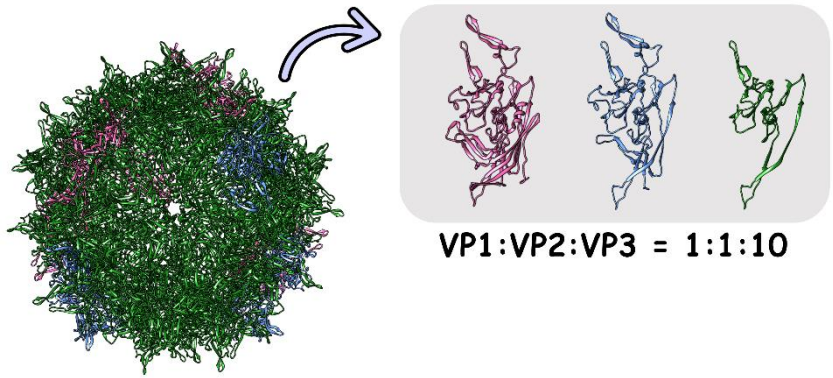


Evaluation of the method from a quantitative perspective:
the area of each peak was additive, linear and specific to the empty and full rAAV

The developed method has the potential to be used in QC environment!!

Aebischer et al. IJMS 2022, 23, 12332

Capsid integrity evaluation (VP1-3 Ratio)



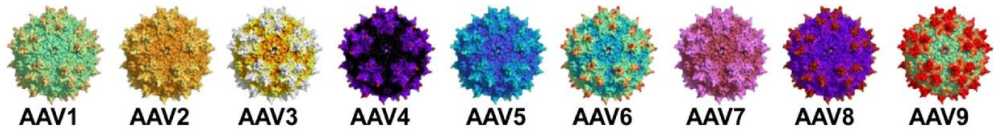
VP1 (735 aa, 87 kDa)

VP2 (596 aa, 73 kDa)

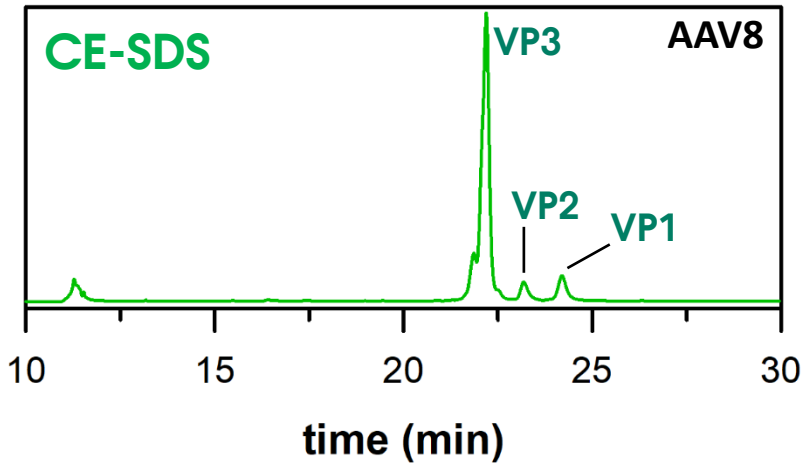
VP3 (533 aa, 62 kDa)

Common PTMs = phosphorylation, deamidation, oxidation, acetylation…

Large sequence homology (51 – 99%) among VPs derived from different AAV serotypes

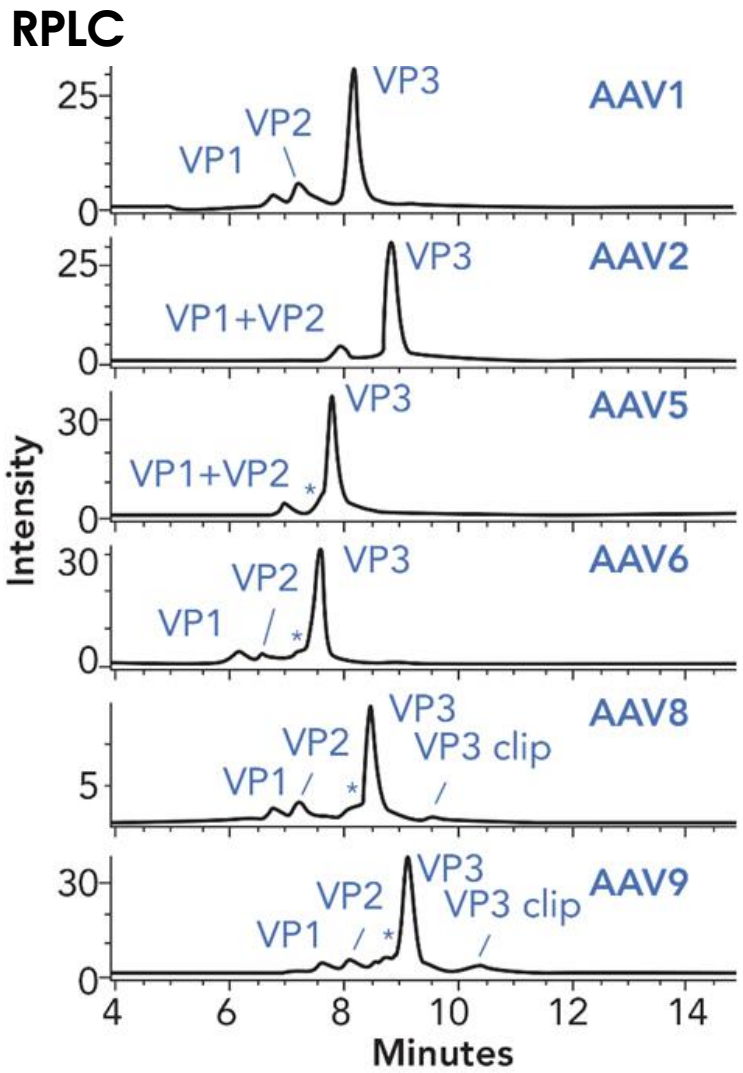


VPs are generally analyzed by CE-based techniques.
CE-SDS/cIEF are routinely applied for their excellent resolving power of the VPs.



CE-SDS = Capillary electrophoresis sodium dodecyl sulfate; cIEF = capillary IsoElectric Focusing

Capsid integrity evaluation by LC-based methods



RPLC mode Waters Protein BEH C4 column (300A, 1,7 um, 2,1x150mm); Gradient 32% to 36%B in 16 min. MPA = 0,1% DFA in water, MPB = 0,1% DFA in ACN. FR = 0,2 mL/min, T = 80°C.

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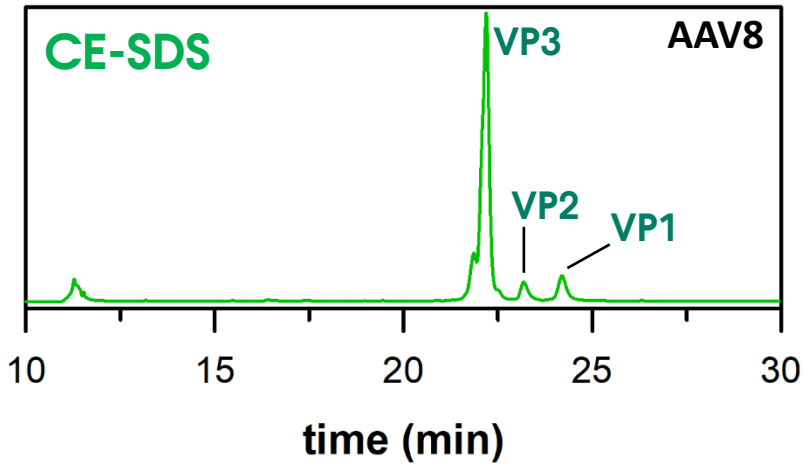


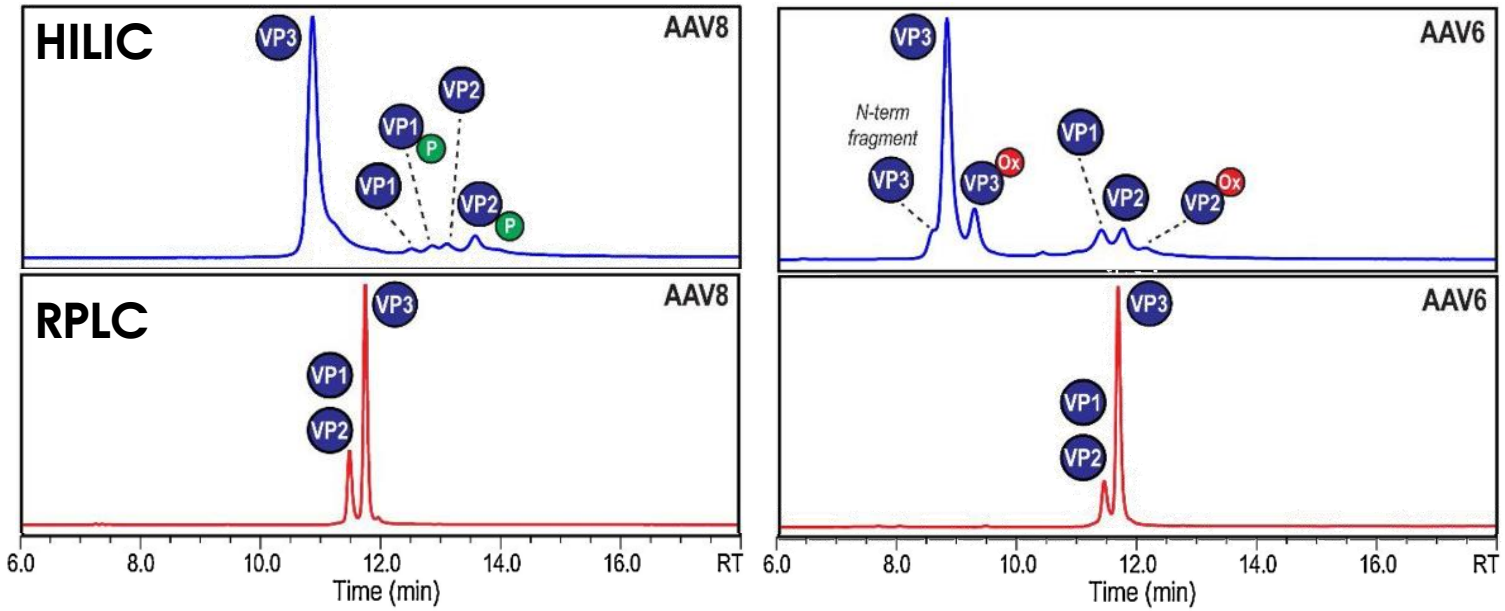
← RPLC/MS is used as valid orthogonal approach!

REF RPLC = Zhang et al. Human Gene Therapy 2021, 32, 1501-1511;
CE-SDS = Capillary electrophoresis sodium dodecyl sulfate; cIEF = capillary IsoElectric Focusing

Improved selectivity by using multi-isocratic elution

HILIC is an extremely interesting option as it has a complementary retention mechanism to RPLC.

Separation of both oxidized and phosphorylated VP proteoforms!



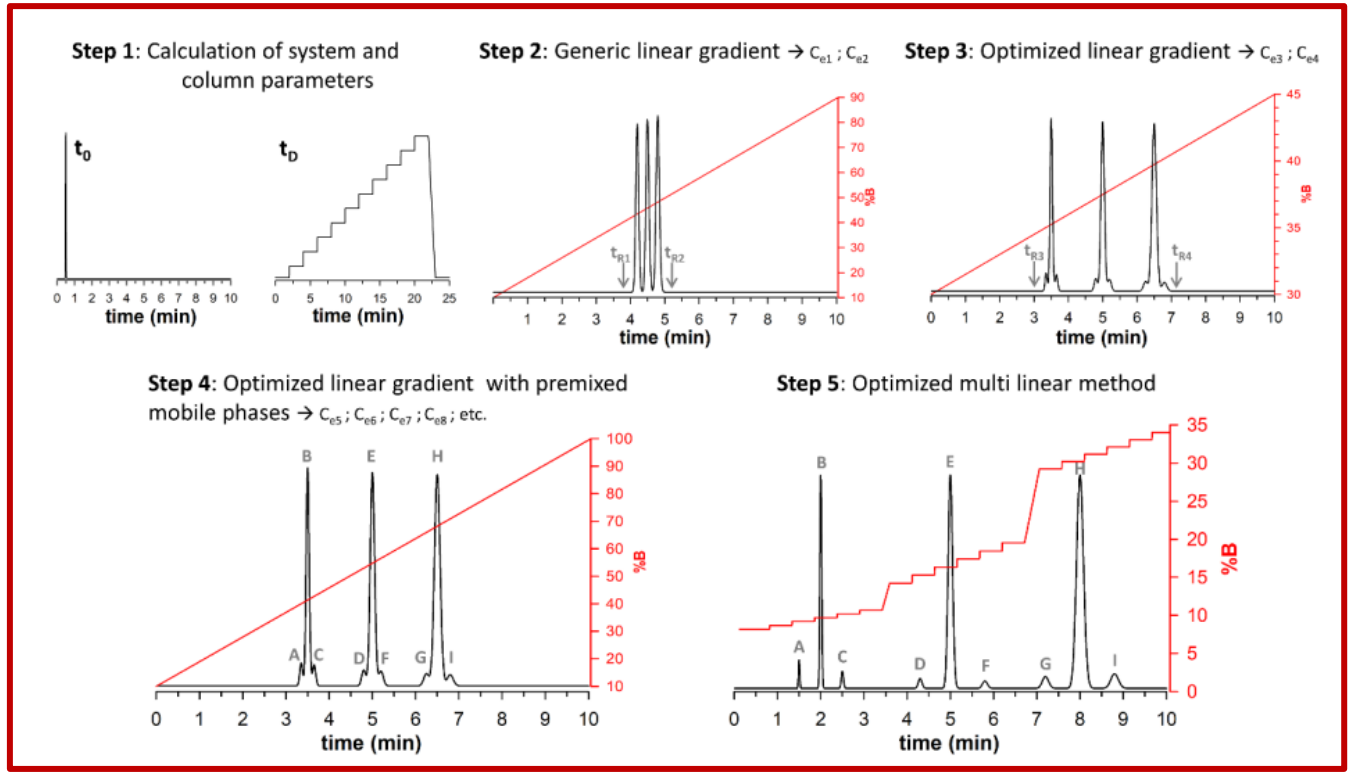
HILIC method is patented!!
US 2020/0131533 A1 (Apr 30, 2020)

REF = Liu et al. JPBA 2020, 189, 113481

Improved selectivity by using multi-isocratic elution

VPs are made of proteins,
do they follow an "on/off" retention behaviour?

Use of the workflow in 5 steps (Murisier et al. Separations 2022, 9, 243)
to develop multi-isocratic elution methods in RPLC, HILIC, and HIC.



Murisier et al. Separations 2022, 9, 243 & Aebischer et al. IJMS 2023, 24, 8503

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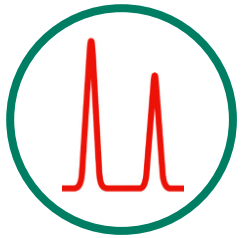

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Patented HILIC mode (Liu et al. JPBA 2020)
Waters Glycoprotein BEH Amide column
(300A, 1.7 um, 2.1 x 150mm);
Injection volume = 3 x 1 µL (multiple loading)
MPA = 0.1%TFA in water
MPB = 0.1%TFA in ACN
T = 60 °C
FR = 0.2 mL/min
Gradient = linear with initial ramp at 85%B

Optimized HILIC mode (Aebischer et al. IJMS 2023)
Waters Glycoprotein BEH Amide column
(300A, 1.7 um, 2.1 x **50mm**);
Injection volume = 0.3 µL
MPA = 0.1%TFA in water
MPB = 0.1%TFA in ACN:IPA 80:20
T = 40°C
FR = 0.4 mL/min
Gradient: multi-step gradient with premixed MPs

Liu et al. JPBA 2020, 189, 113481 & Aebischer et al. IJMS 2023, 24, 8503

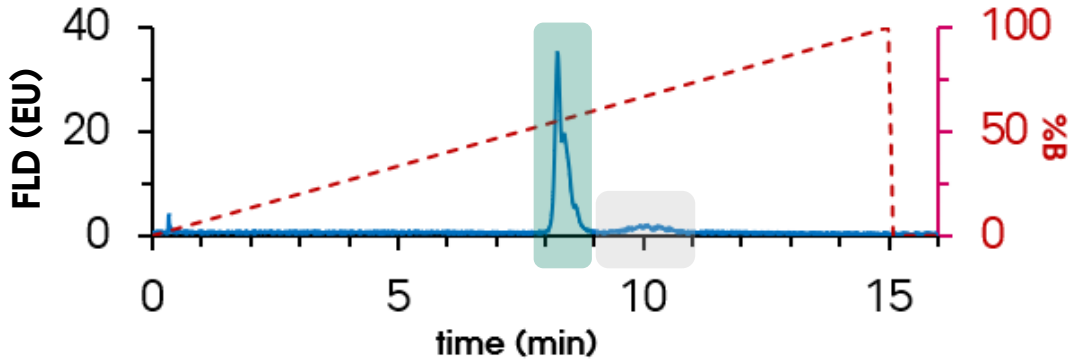
Method development in HILIC mode: optimized linear gradient

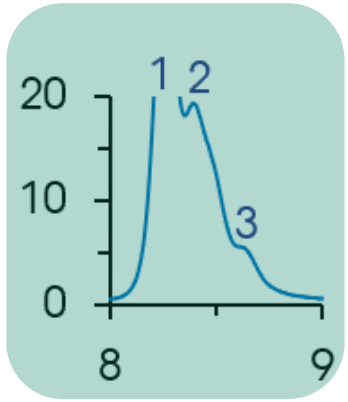
A = [ACN/IPA, 80/20] + 0.1% TFA / H2O + 0.1% TFA (79/21)
B = [ACN/IPA, 80/20] + 0.1% TFA / H2O + 0.1% TFA (67/33)

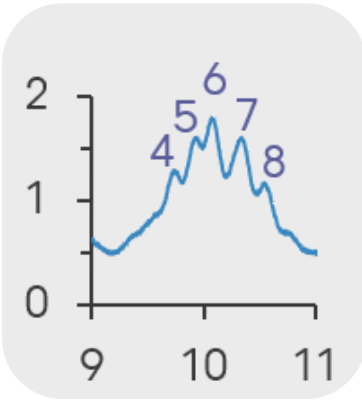
15 min | 40 °C | 0.4 mL/min

1. Generic linear gradient with premixed MPs

Time (min)	% B
0	0
15	100
15.1	0
20	0

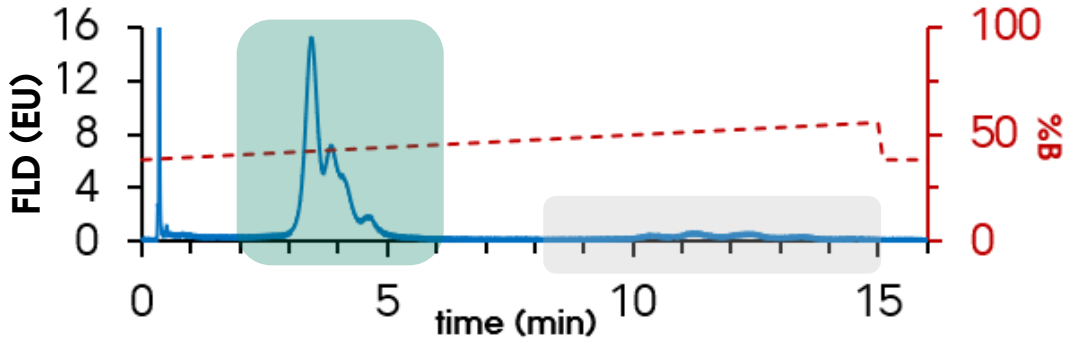


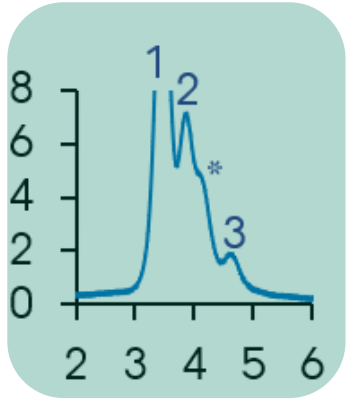


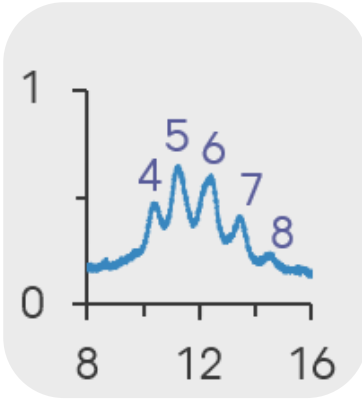


2. Optimized linear gradient with premixed MPs

Time (min)	% B
0	38
15	56
15.1	38
20	38





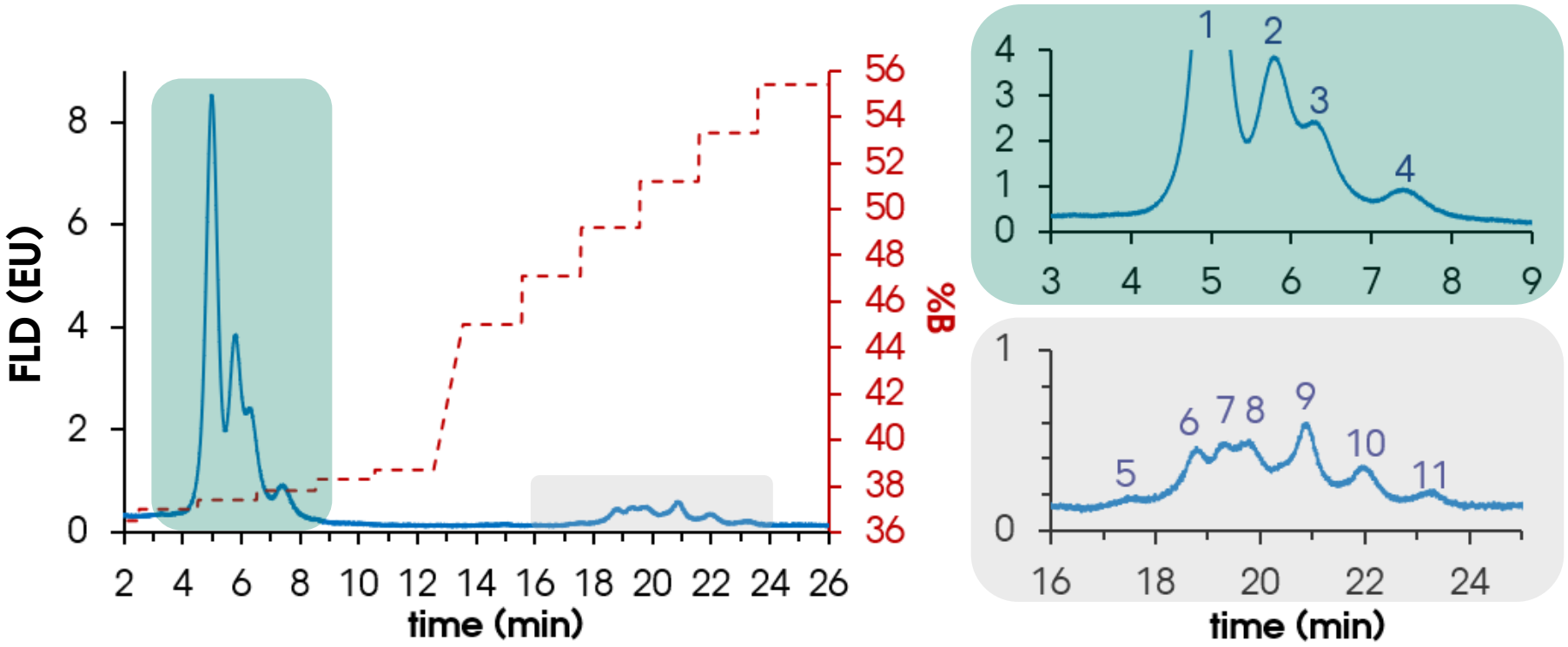


Aebischer et al. IJMS 2023, 24, 8503

Method development in HILIC mode: multi-step gradient elution mode

3. Multi-step gradient with premixed MPs

Time (min)	% B
0	36.5
0.50	36.5
2.50	36.5
2.51	37.0
4.51	37.0
4.52	37.4
6.52	37.4
6.53	37.8
8.53	37.8
8.54	38.3
10.54	38.3
10.55	38.7
12.55	38.7
13.55	45.0
15.55	45.0
15.56	47.1
17.56	47.1
17.57	49.2
19.57	49.2
19.58	51.2
21.58	51.2
21.59	53.3
23.59	53.3
23.60	55.4
26.10	55.4
26.20	36.5
40	36.5

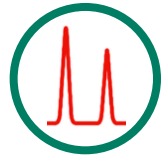


Separation of 11 viral protein variants at chromatographic level!

Aebischer et al. IJMS 2023, 24, 8503

Conclusions

Improved selectivity by using multi-isocratic elution



- Feasible for rAAV capsids
- Feasible for rAAV VPs
- Best selectivity gain when limited number of peaks to deal with (as for rAAV capsids application)
- Complex gradient program when dealing with large number of peaks (as for rAAV VPs application)

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