

Introduction to HPLC Separation Principles for Biomolecules

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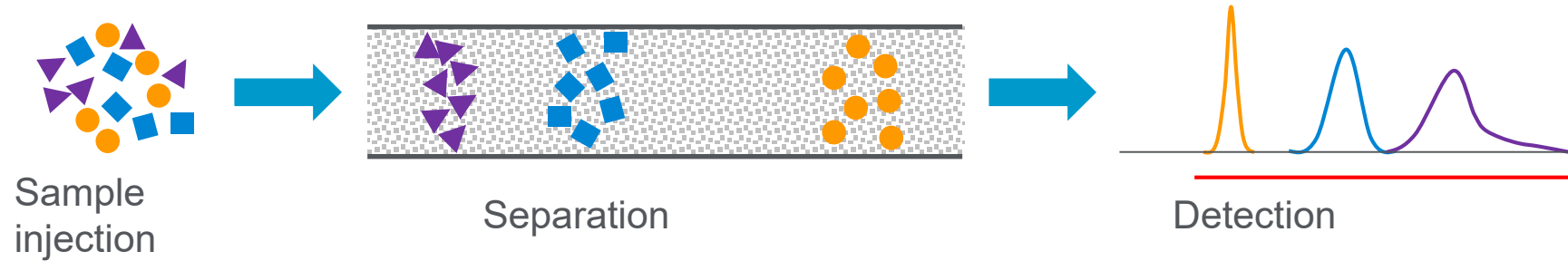
Introduction to HPLC Separation Principles for Biomolecules

Overview

- Principles of LC Separation
- Stationary Phase Particle Development
- Separation Modes

Introduction to LC

What is LC?



- Analyte elution delayed by different retention times due to varying affinity for the stationary and mobile phases
- Minimize peak width to increase peak capacity and resolution
- Column behavior determined by
 - Dispersion / peak width (column geometry, packing uniformity, intraparticle diffusion)
 - Retention (mobile-phase partitioning, chemical interaction)

Dustin Chang, August 2011

Resolution ...

Determined by 3 Key Parameters – Retention, Selectivity and Efficiency

k = Retention Factor –

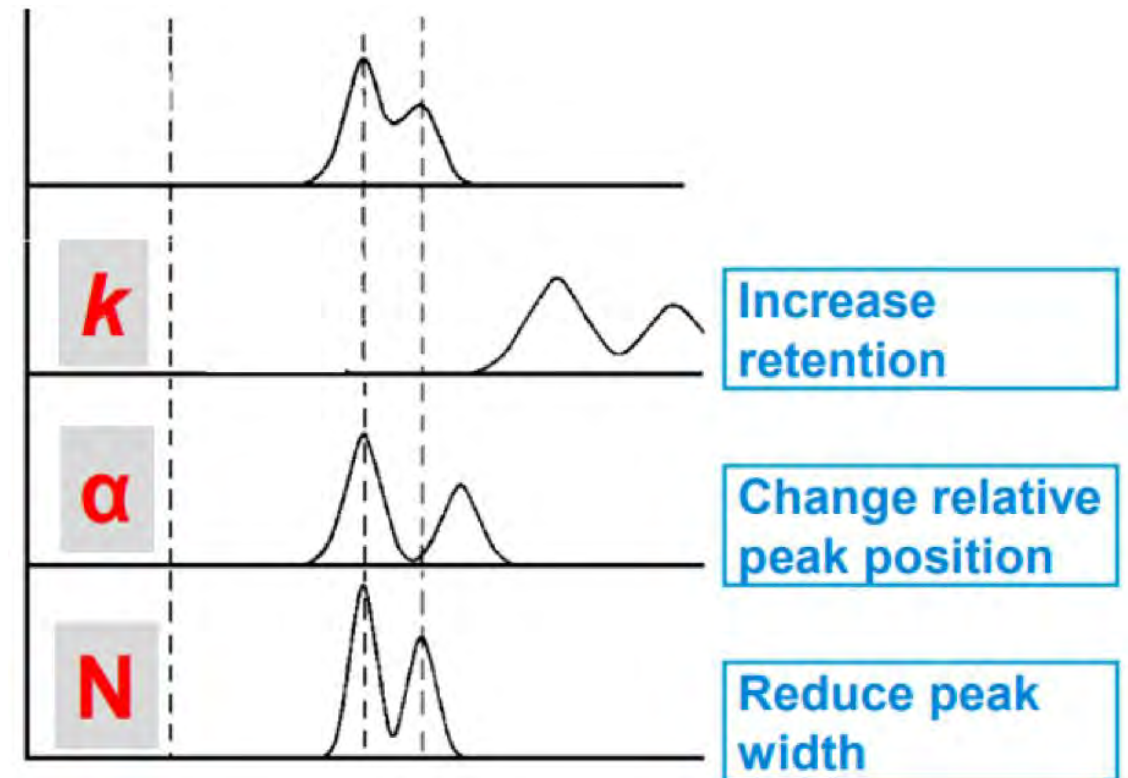
Mobile phase strength, stationary phase, temperature

α = Selectivity –

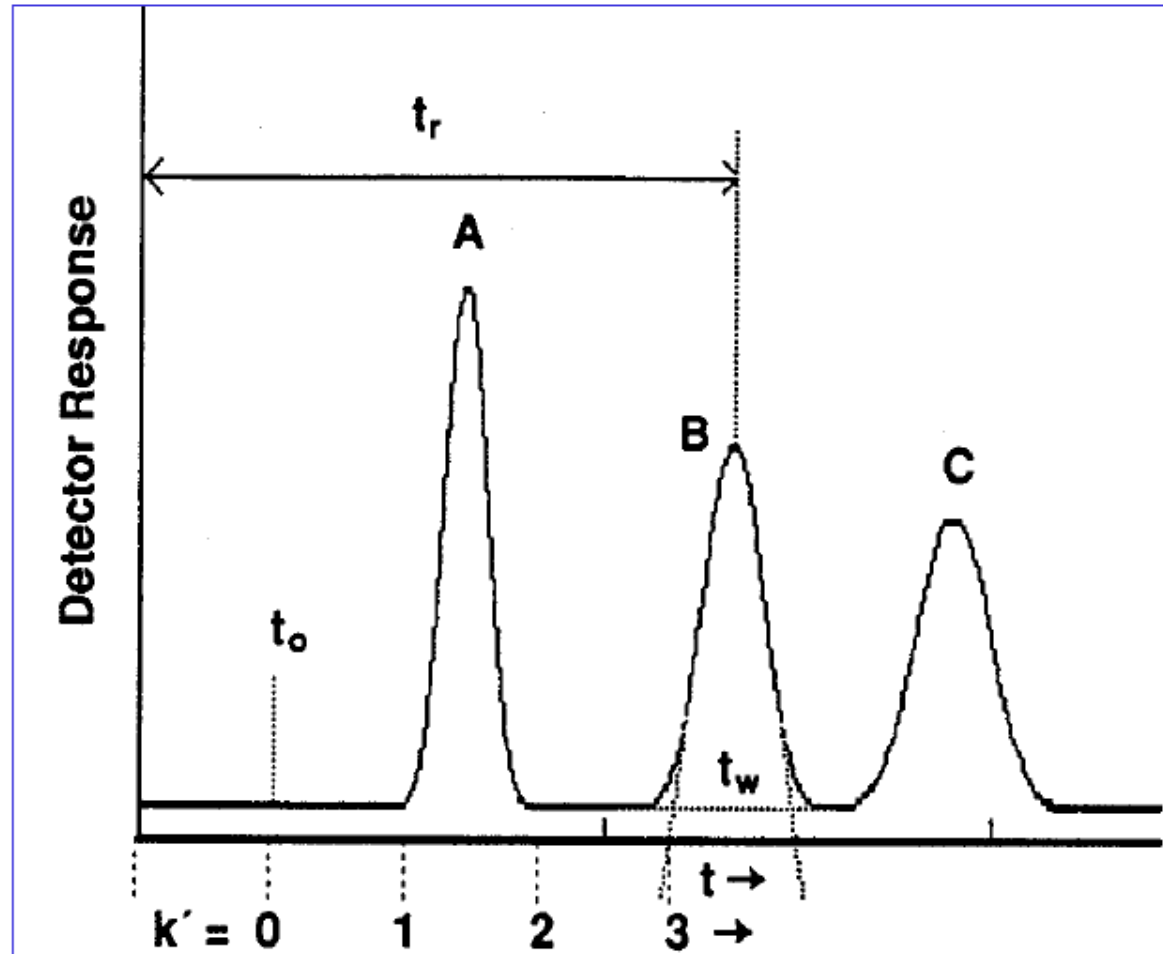
Mobile phase, stationary phase, mobile phase pH

N = Column Efficiency –

Physical parameters:
Column length, particle size, particle morphology
low dispersion LC systems



Equations Describing Separation



Resolution: $R_s = \frac{t_{R-2} - t_{R-1}}{(w_2 + w_1)/2} = \frac{\Delta t_R}{\bar{w}}$

Retention Factor: $k = \frac{(t_R - t_0)}{t_0}$

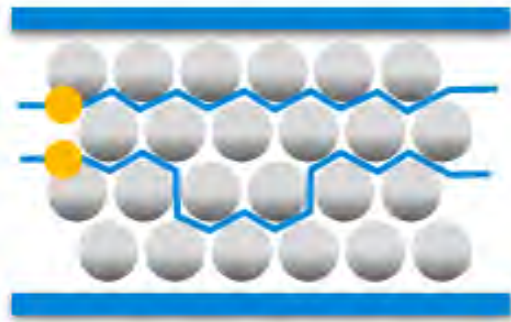
Selectivity: $\alpha = k_2/k_1$

Number of Theoretical Plates (Efficiency): $N = 16(t_R / t_{W-base})^2$

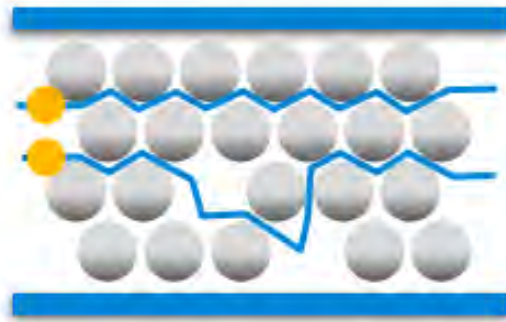
Plate Height: $h = \frac{\text{Column Length}}{N}$

Analyte Diffusion Effects Column Efficiency (peak sharpness):

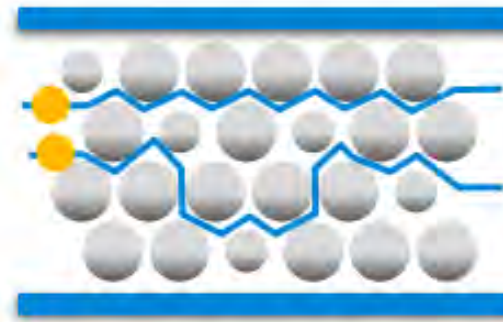
1) Eddy Diffusion – Differences in diffusion paths due to:



Different paths

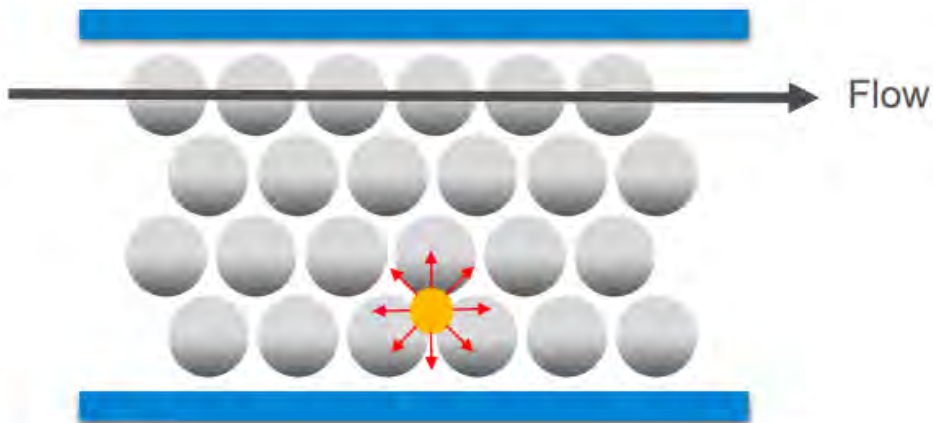


Poor column packing

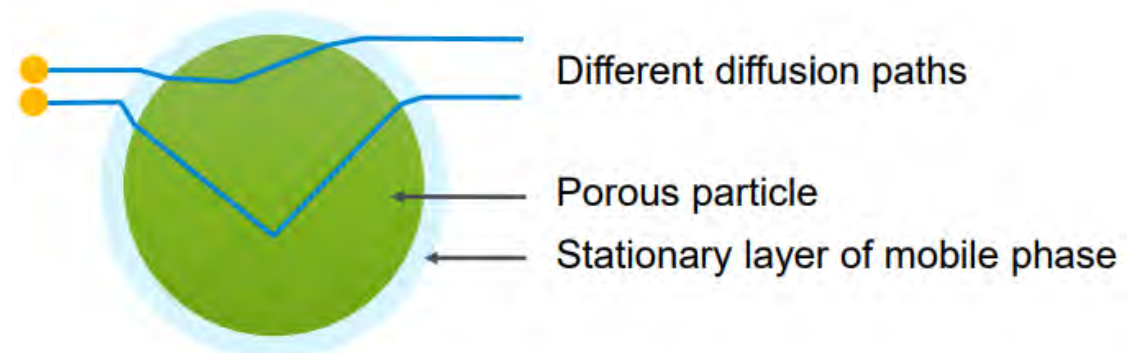


Broad particle size distribution

2) Axial or Longitudinal Diffusion:

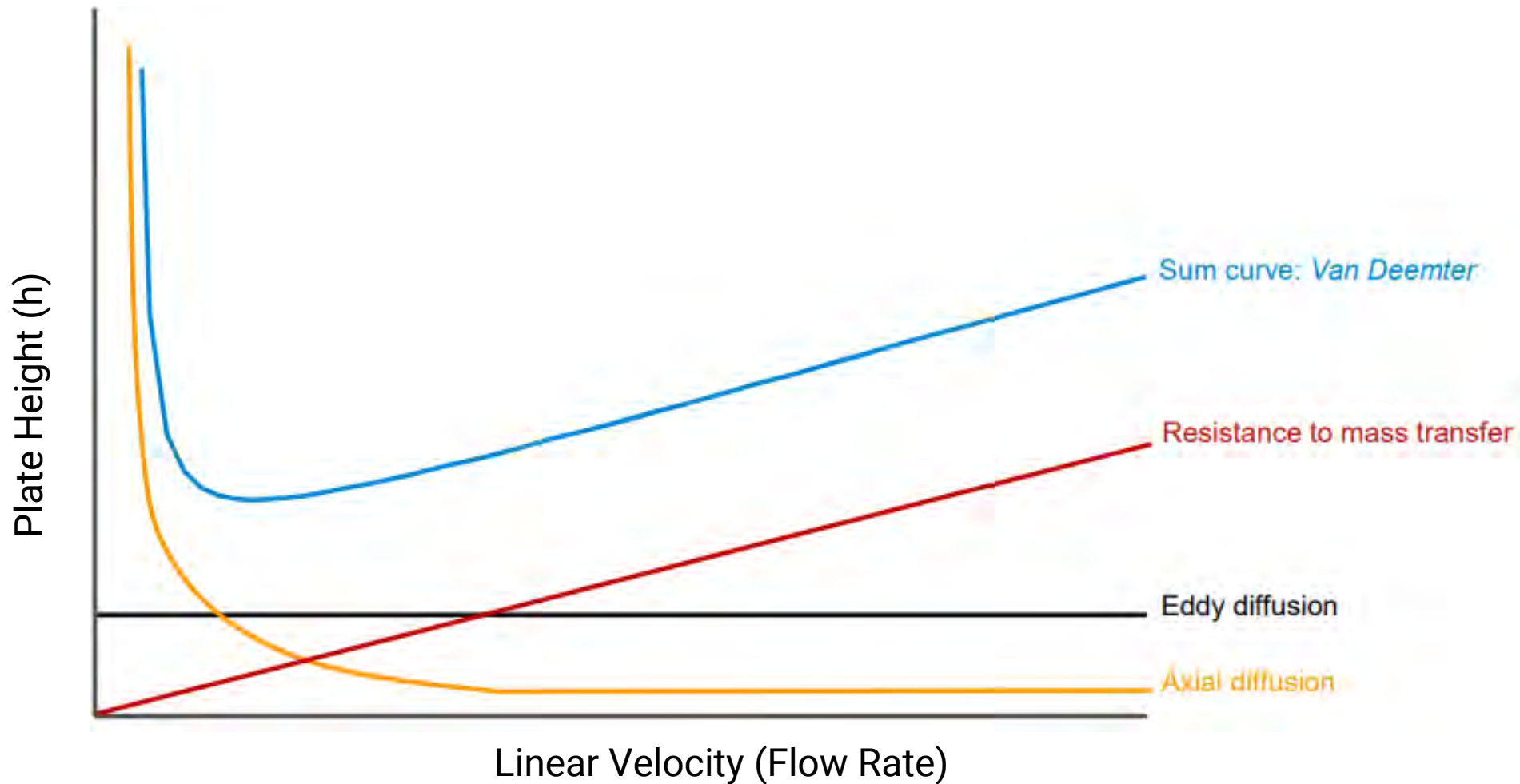


3) Resistance to Mass Transfer - Differences in diffusion paths within a particle:



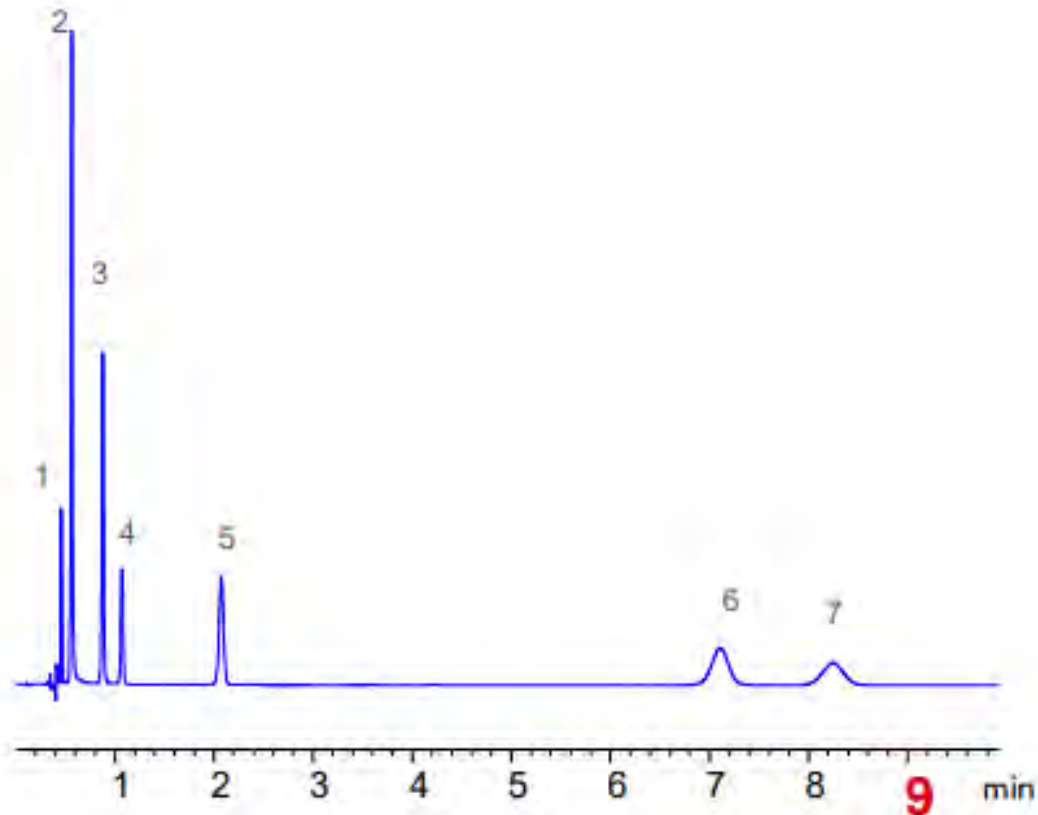
Van Deemter Plot – Effect of Flow Rate on Efficiency (Flow Rate Effects Analyte Diffusion)

Height of Theoretical Plate (h) = f (Eddy diffusion + Axial diffusion + Resistance to mass transfer)

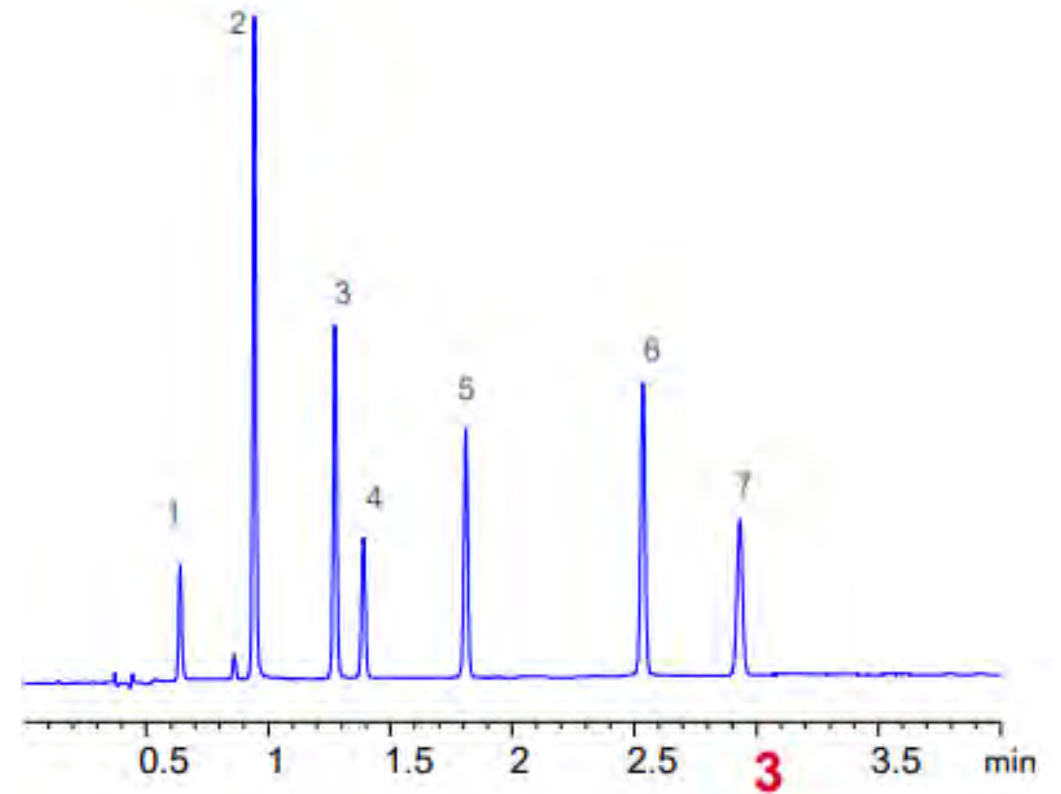


Isocratic vs Gradient Elution

Isocratic: Same mobile phase throughout run



Gradient: Continuously increasing the solvent strength of the mobile phase



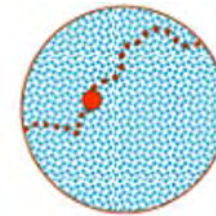
History of HPLC Particle Development

Adapted from R.E. Majors, HPLC 2013

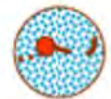
Year(s) of Acceptance	Particle Size	Most Popular Nominal Size
1950's		100 μm
1967		50 μm
1972		10 μm
1985		5 μm
1992		3-3.5 μm
1999		5.0 μm superficially porous (300Å)
1999		5.0 μm 1 st generation hybrid silica
2000		2.5 μm
2003		1.8 μm
2004		1.7 μm 2 nd generation hybrid silica
2007		2.7 μm superficially porous (100Å)
2016		1.9 μm superficially porous (120Å)

Physical Properties of Chromatography Columns

- Column dimensions
 - Longer columns can give better separation but longer run time
 - Smaller ID columns use less mobile phase and sample but are less forgiving of extra column volume
- Particle size
 - Smaller particles allow quicker diffusion from stationary phase to mobile phase and sharper peaks
 - Smaller particles increase backpressure and are more subject to clogging



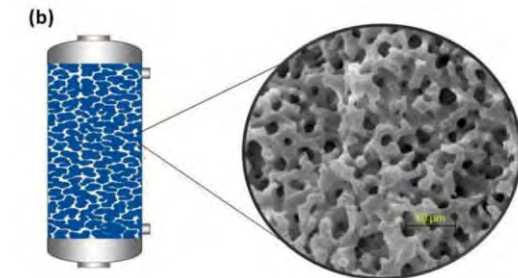
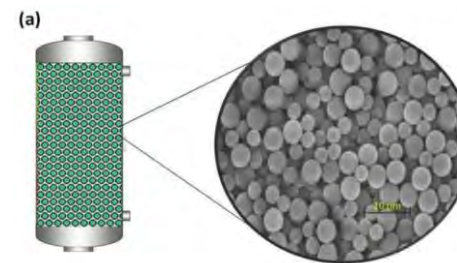
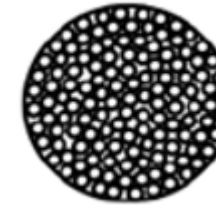
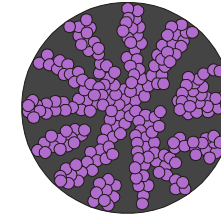
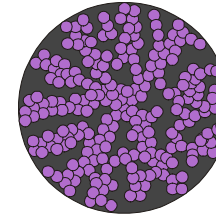
**Large
Particle**



**Small
Particle**

Physical Properties of Chromatography Columns

- Pore size
 - Appropriate pore size is needed for size of analyte so analyte can easily diffuse into and out of pores
- Superficially porous particles (SPP) vs totally porous particles (TPP) vs non porous (NP)
- Packed spherical particles vs monolith



Column Backpressure

- Equation for Pressure Drop Across an HPLC Column:
$$\Delta P = \frac{\eta \cdot L \cdot v}{d_p^2}$$
- **Small particles create high backpressure!**
 - Backpressure is highly dependent on particle size
 - Ultra High Performance (Pressure) Liquid Chromatography - UHPLC
- Backpressure is proportional to the length of the column
- Backpressure increases with flow rate
- Solvent viscosity affects backpressure – solvent viscosity can change considerably if mobile phase composition is changed during a gradient elution
- Solvent temperature affects viscosity
- Columns have limits to backpressure before column bead is crushed leaving a void in the column - check column documentation
- Know the expected backpressure of your column and system when functioning normally
 - Helpful for troubleshooting issues

ΔP = Pressure Drop

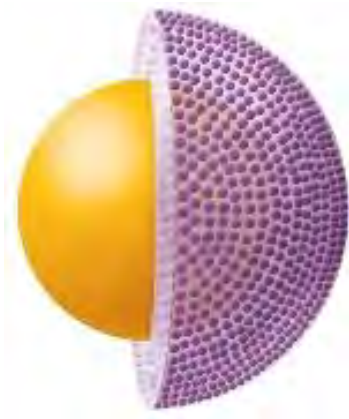
η = Fluid Viscosity

L = Column Length

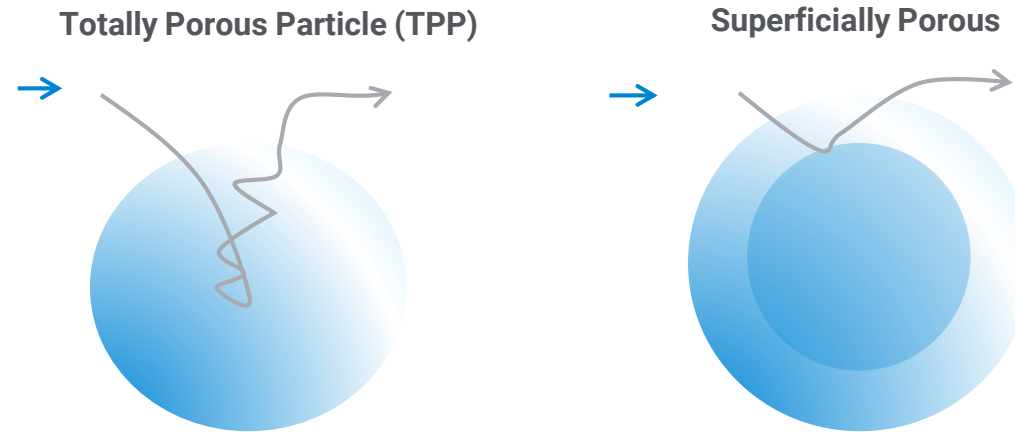
v = Flow Velocity

d_p = Particle Diameter

Superficially Porous Particle Technology – What makes it better?



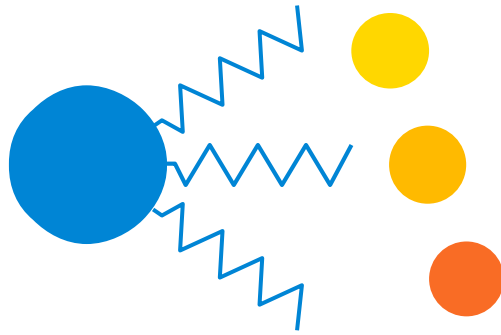
Poroshell is made of a solid core with a porous outer layer



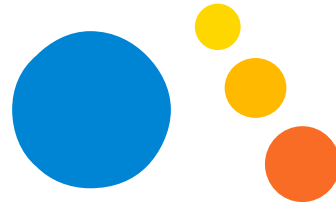
- Analytes travel through the particle more efficiently: improving peak shape and resulting in faster run-times
- High efficiency allows you to use a larger SPP (ie. 2.7 μ m) for nearly equivalent performance to a smaller TPP column (ie. sub-2 μ m)
- Using a larger particle allows for lower backpressure than comparable TPP columns, and flexible use on HPLC or UHPLC systems

Chromatographic Techniques used in Biopharma

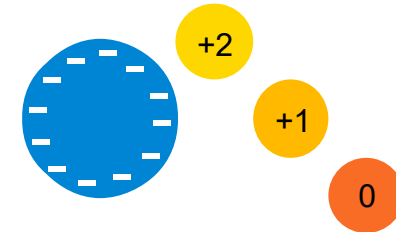
Reversed Phase



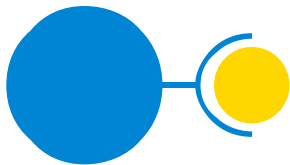
Size Exclusion



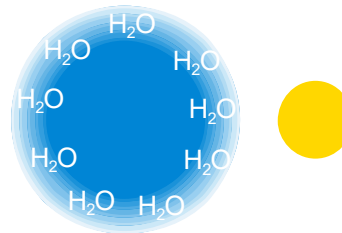
Ion Exchange



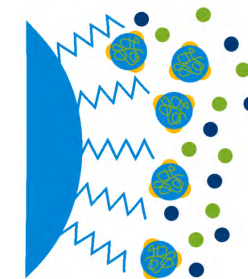
Affinity



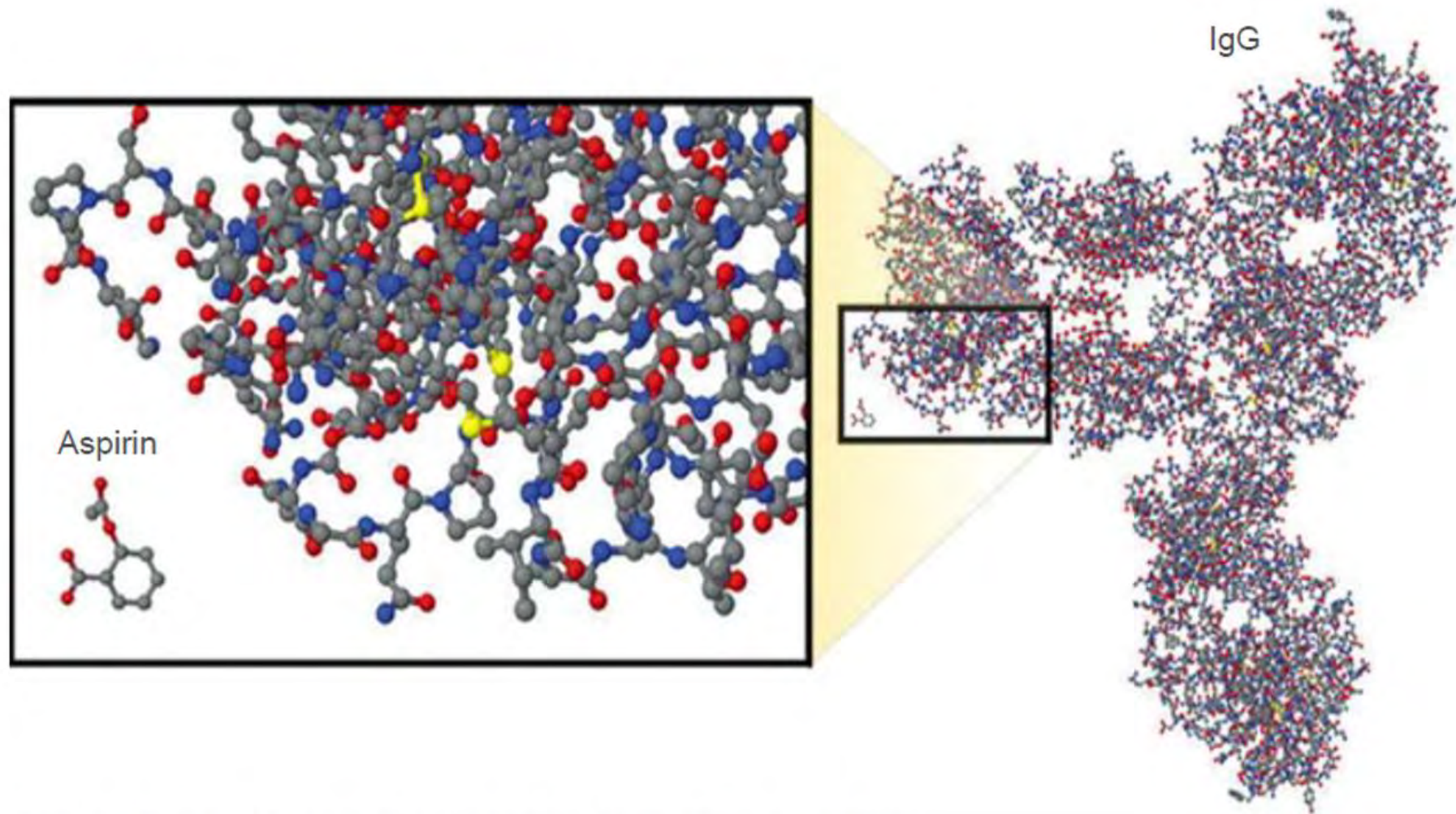
Hydrophilic Interaction Chromatography (HILIC)



Hydrophobic Interaction Chromatography (HIC)

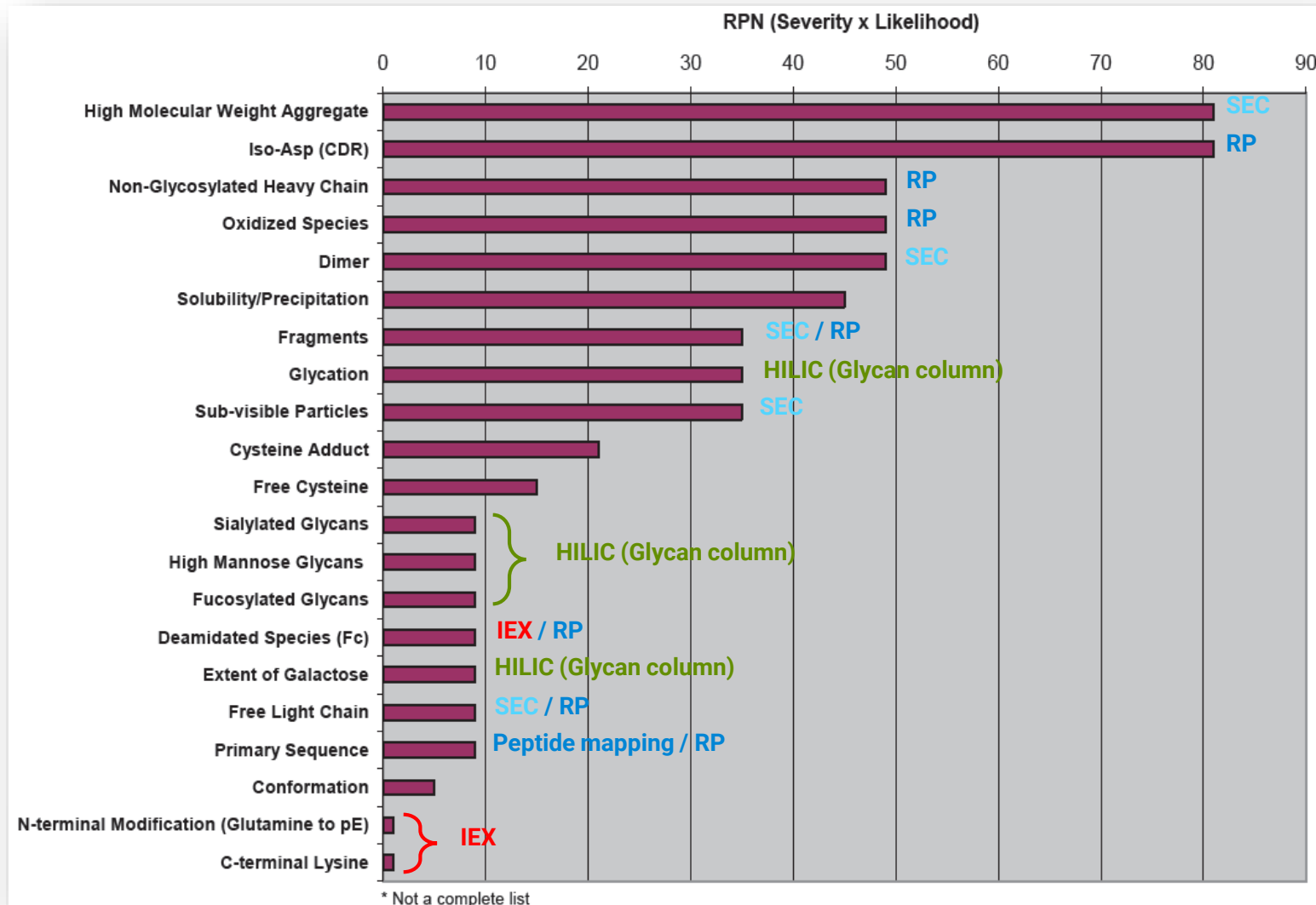


Complexity of a mAb vs Small Molecule Drug

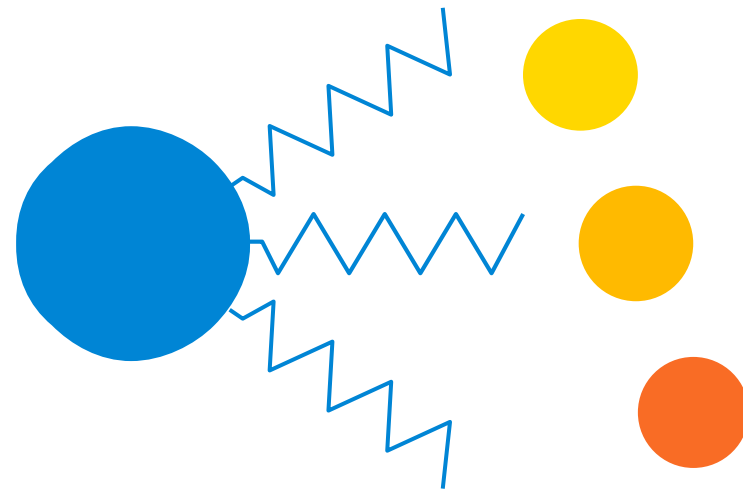


Source – New England Journal of Medicine, "Developing the Nation's Biosimilars Program," August 4, 2011

Range of Critical Quality Attributes



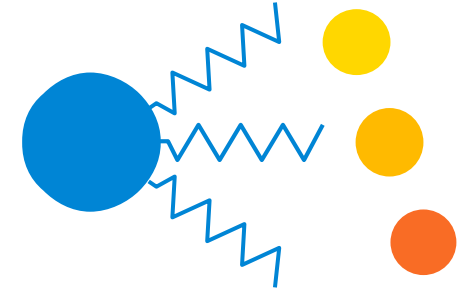
Reversed Phase Separations



Reversed Phase Chromatography (RPLC)

Principle: Partition of analytes between polar mobile phase and non-polar stationary phase

- Adoption of reversed phase chromatography followed development of normal phase chromatography that used polar stationary phases, such as bare silica, with non-polar mobile phase
- Non-polar interactions of analyte with hydrophobic (or lipophilic) stationary phase:
 - C18, C8, Phenyl, C3, etc.
- Different sorption affinities between analytes results in their separation
 - More polar analytes are less retained
 - Analytes with larger hydrophobic part are retained longer
- Mobile phase: water (buffer) + water-miscible organic solvent e.g. MeOH, ACN

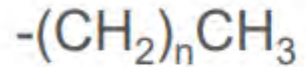


Nonpolar and Polar Functional Groups*

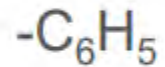
Nonpolar (Hydrophobic)



- Methyl



- Alkyl groups

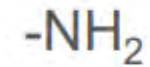


- Phenyl groups

Polar (Hydrophilic)



- Carboxyl groups



- Amino groups



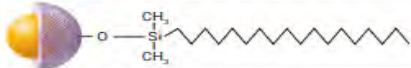
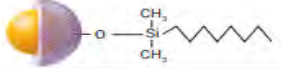
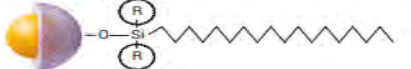
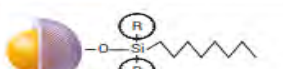
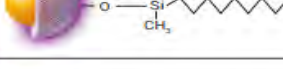
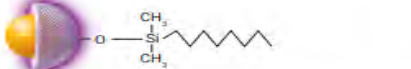
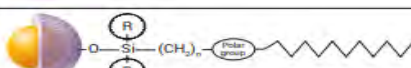
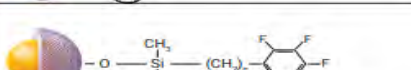
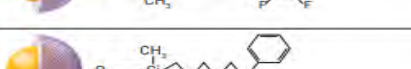
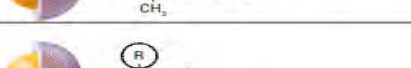
- Hydroxyl Groups



- Amide groups

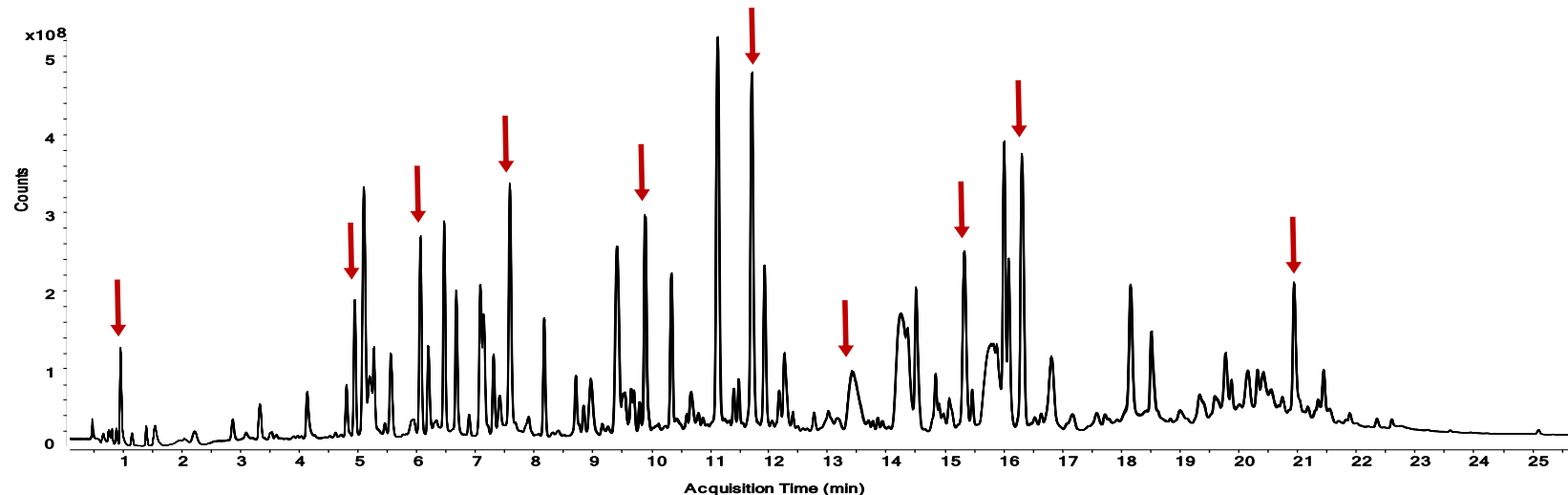
*Affect solubility and elution order

Many Bonded Phases for Reversed Phase Columns

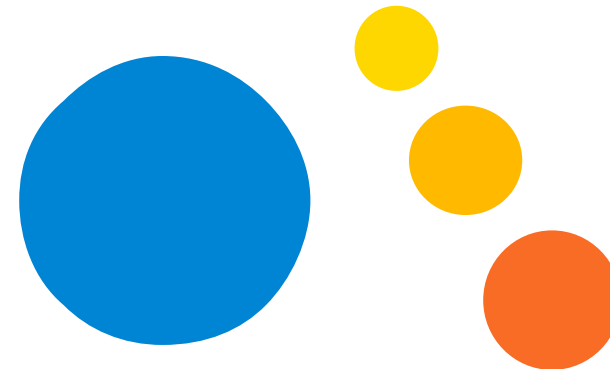
InfinityLab Poroshell 120	Chemistry	Particle Sizes	Benefits and Applications
EC-C18		1.9, 2.7, 4 µm	General purpose Excellent peak shape and efficiency for acids, bases, and neutrals
EC-C8		1.9, 2.7, 4 µm	General purpose Lower retention of hydrophobic analytes vs. C18
SB-C18		1.9, 2.7, 4 µm	Low pH Excellent stability and peak shape in highly acidic conditions
SB-C8		2.7 µm	Low pH Excellent stability at low pH Lower retention of hydrophobic analytes vs. C18
HPH-C18		1.9, 2.7, 4 µm	High pH capable Robust performance and long lifetimes Improved retention, resolution, and peak shape of basic compounds
HPH-C8		2.7, 4 µm	High pH capable Robust performance and long lifetimes Lower retention of hydrophobic analytes vs. C18
Bonus-RP		2.7 µm	Alternative selectivity to C18 Improved peak shape for basic compounds, stable in 100% aqueous conditions
PFP		1.9, 2.7, 4 µm	Alternative selectivity Excellent peak shape for polar and nonpolar analytes Unique selectivity for aromatic and halogenated compounds
Phenyl-Hexyl		1.9, 2.7, 4 µm	Alternative selectivity with aromatic groups Highly nonpolar bonded phase takes advantage of pi-pi interactions
SB-Aq		1.9, 2.7, 4 µm	Alternative selectivity Excellent peak shape and retention of polar compounds using reversed-phase LC Exceptional stability under high-aqueous conditions, including 100% water
EC-CN		2.7 µm	Alternative selectivity Use in reversed phase for alternative selectivity of polar and midpolar compounds Use in normal phase for excellent peak shape and retention of nonpolar analytes

Reversed Phase Advantages and Applications in Biopharma

- Allows for highly efficient separations
- Separate denatured proteins, peptides and oligos from impurities
- Compatible with mass spectrometry
 - Intact mass determination
 - Peptide mapping
 - Elucidation of primary structure and modifications to molecule of interest
- Can be used for other applications in biopharma such as desalting and derivatized amino acid analysis

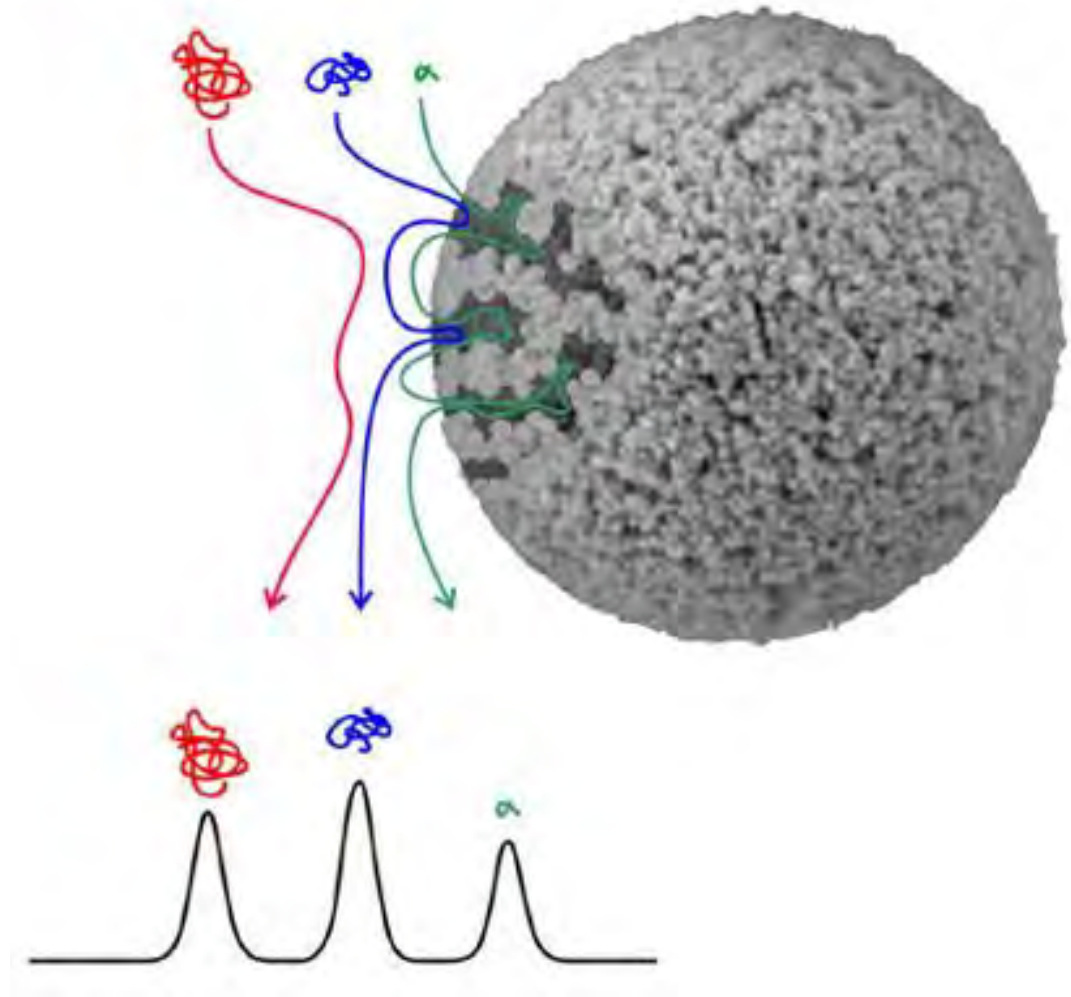


Size Exclusion Separations



Size exclusion Chromatography (SEC) - Mechanism

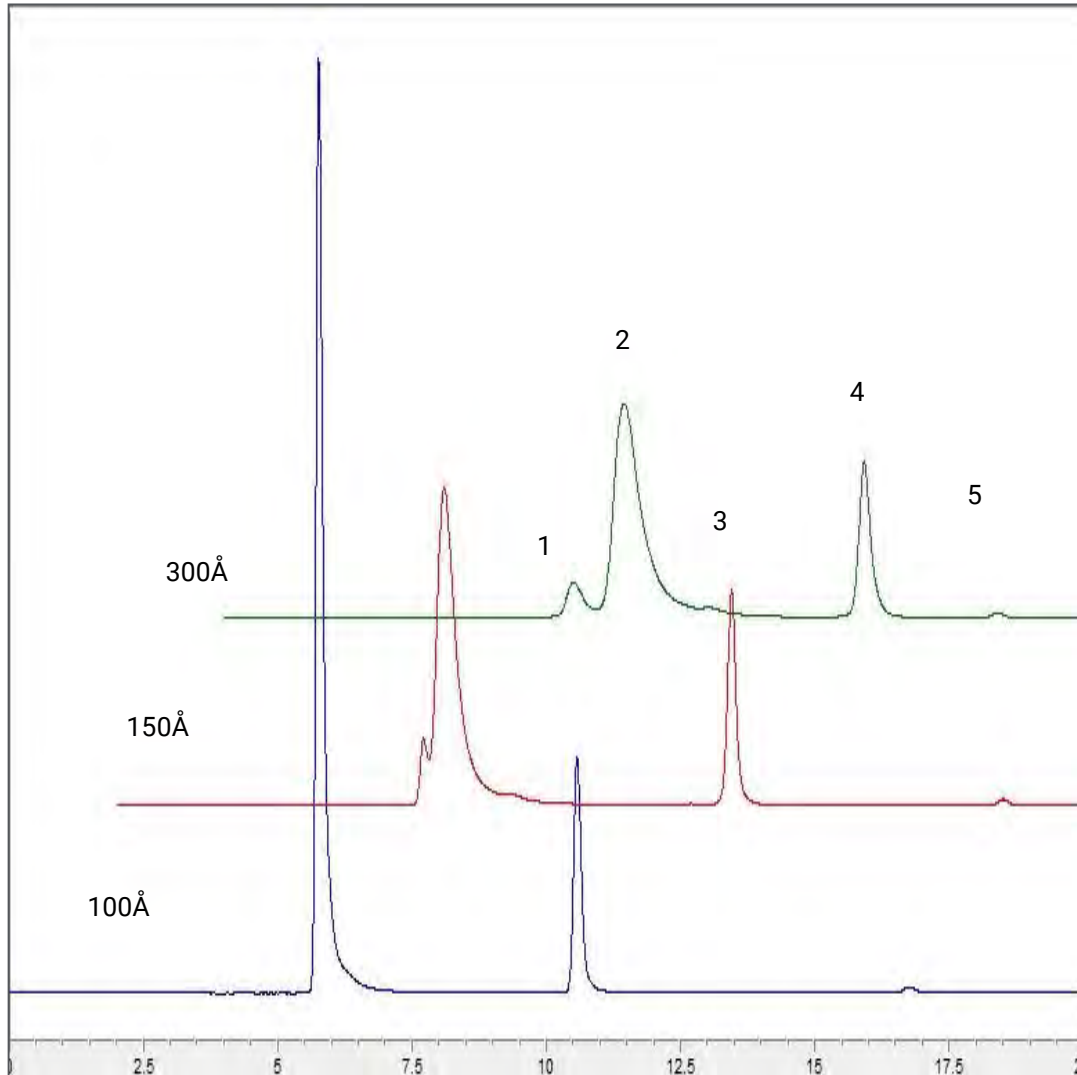
- Largest molecules cannot penetrate the pores – they are excluded and elute first from the column
- Smallest molecules diffuse into all of the pore structure and elute from the columns last
- The resolution of the column is therefore dependent on the pore size and pore volume in the column.
- Separates based on size in solution
- Isocratic



Size exclusion Chromatography (SEC) – Applications and Advantages

- SEC is the standard method for aggregate and fragment analysis of biopharmaceuticals
- Proteins aggregate!
- Protein aggregates greatly enhance immune responses to the protein therapeutic
- These responses may impact safety and efficacy or cause an adverse event
- Protein aggregates can be induced under forced conditions and can occur simultaneously during biopharmaceutical manufacturing
- Non-denaturing – can be used for purification

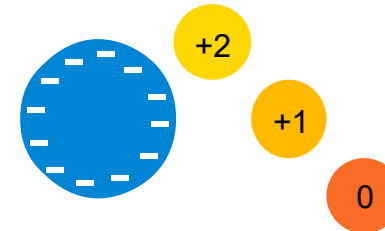
Effect of Pore Size on SEC Separation – Monoclonal Antibody



1. Dimer
2. Monomer
3. Monomer Fragment
4. Azide
5. Retained Molecule

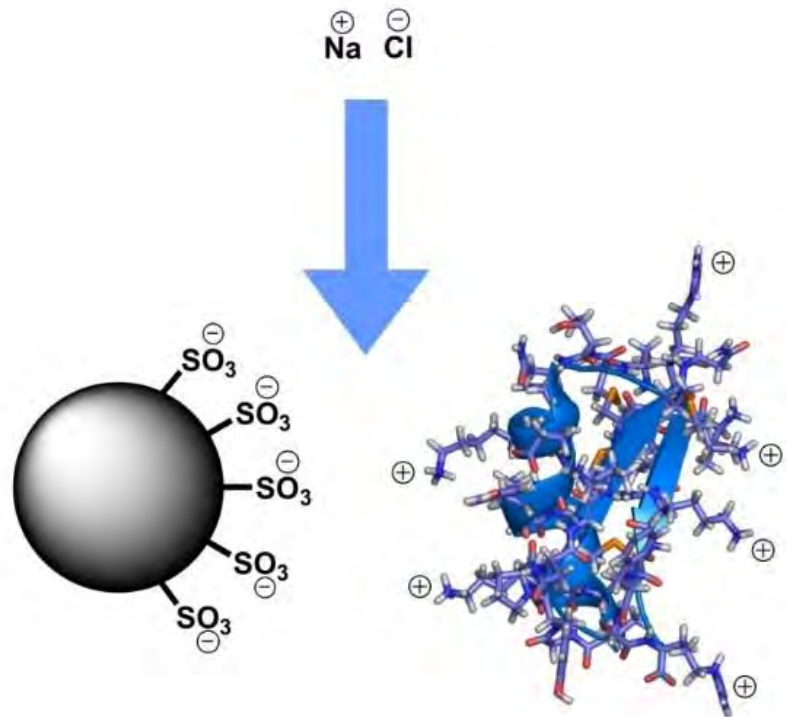
Eluent: 50mM NaH_2PO_4 + 0.15M NaCl, pH6.8
Columns: Agilent Bio SEC-3 ,4.6x300mm
Flow: 0.35ml/min
Detector: UV@220nm
System: Agilent 1260 Infinity Bio-Inert LC
Sample: Mouse IgG

Ion Exchange Separations

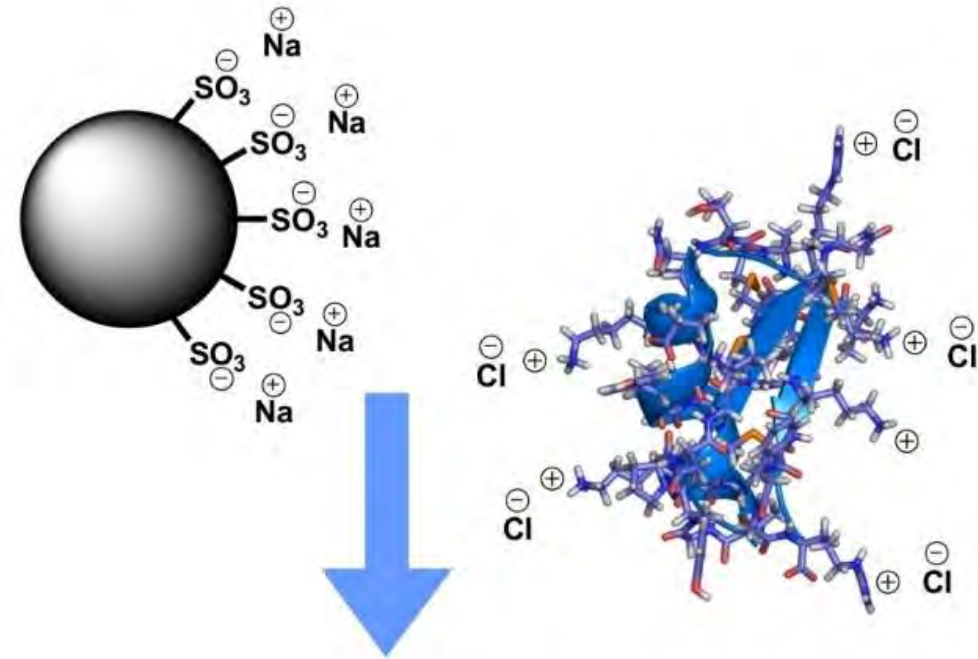


Ion Exchange Chromatography Mechanism

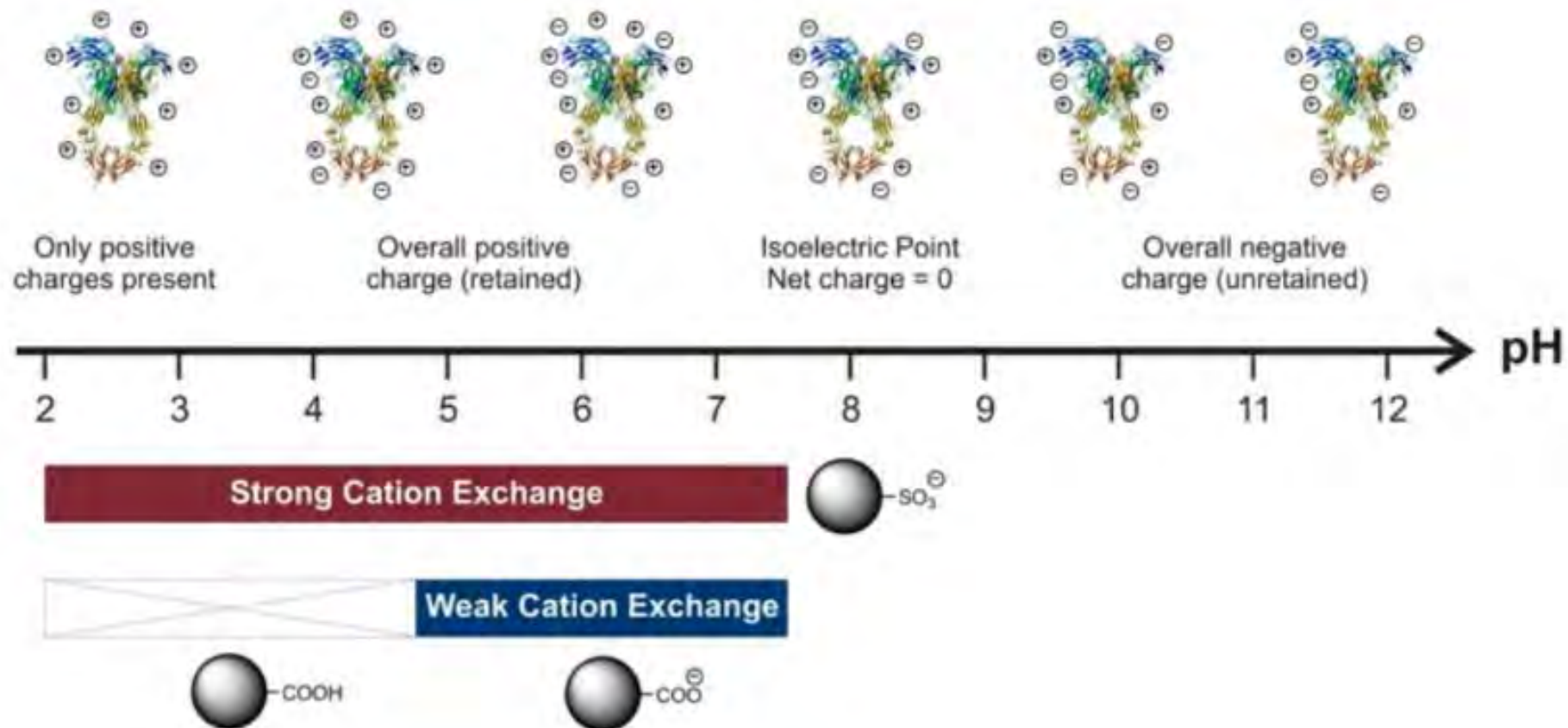
Low salt to bind



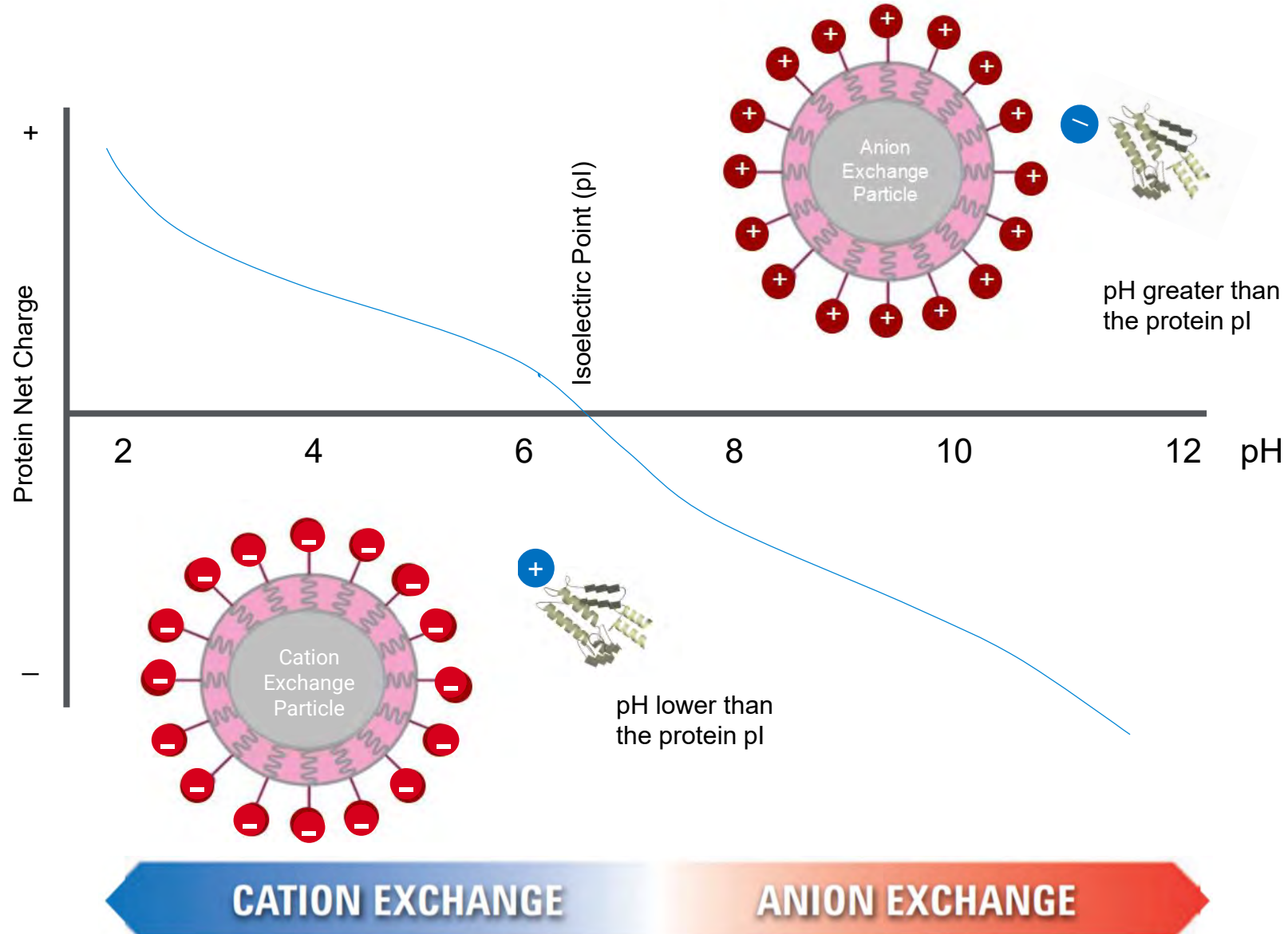
High salt to elute



Effect of pH on Protein Charge for Cation Exchange

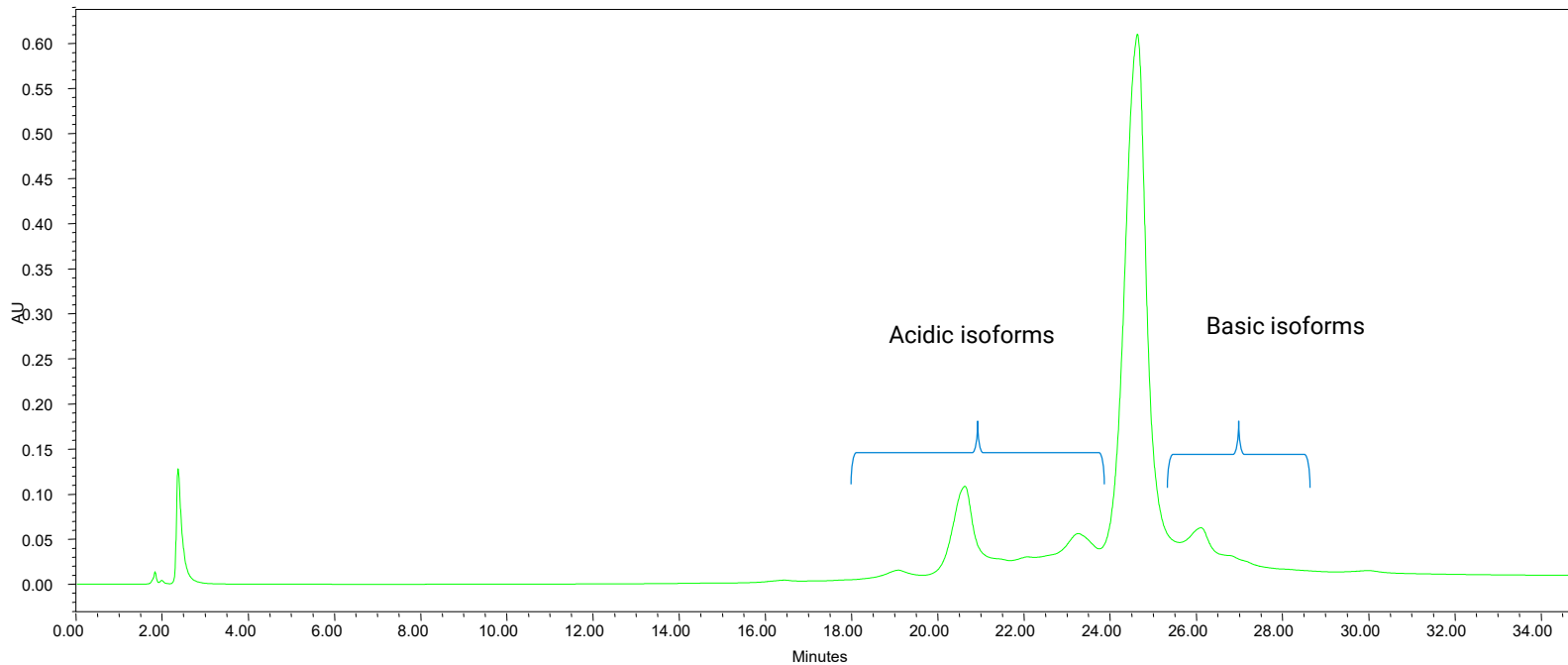


Anion Exchange vs Cation Exchange



Ion Exchange Chromatography Advantages and Applications

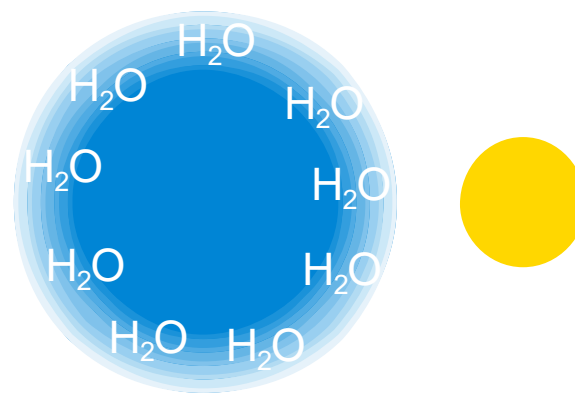
- Non-denaturing - native state analysis of proteins
- Used for prep/purification of proteins, oligonucleotides
- Charge variant analysis



Column: Agilent Bio MAb NP5, 4.6 x 250 mm
Eluent A: 10 mM Na phosphate, pH 7.5
Eluent B: A + 100 mM NaCl, pH 7.5
Gradient: 15-95% B in 60 min
Flow rate: 0.8 mL/min.
Injection: 5 μ L, 5 mg/mL, mAb
Detection: UV, 220 nm

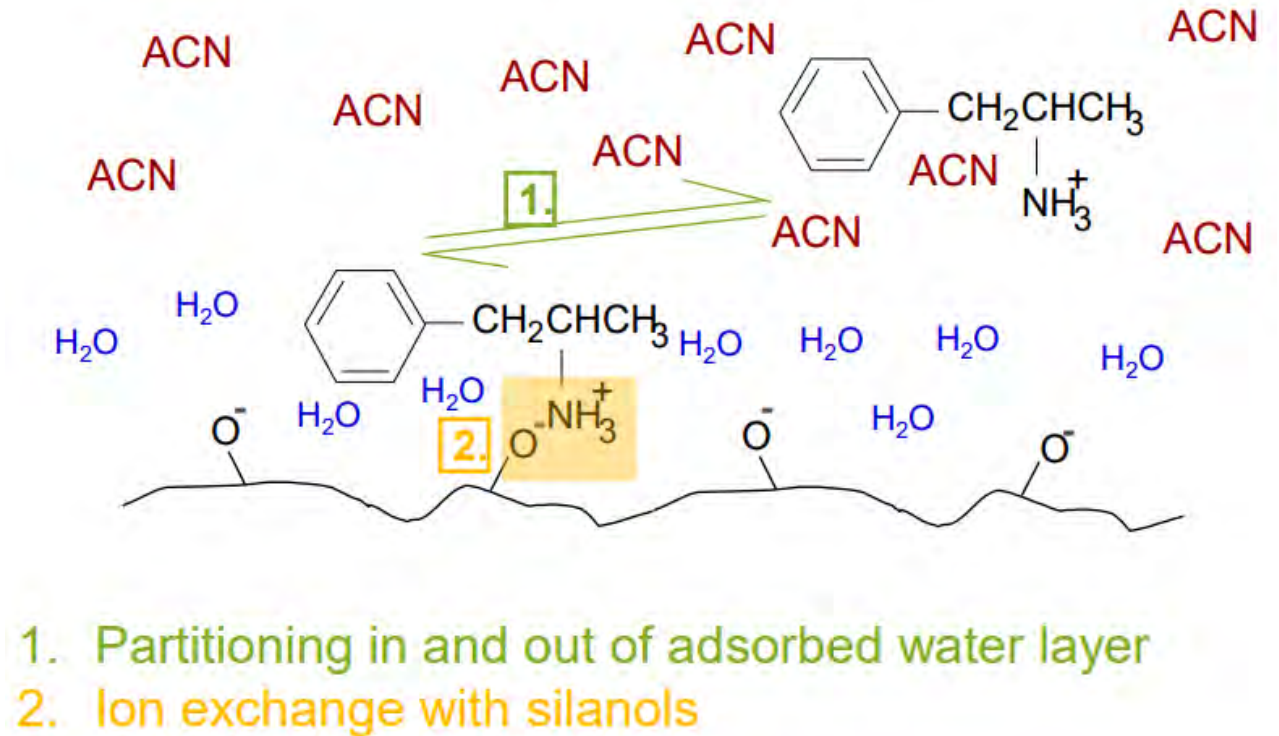
Quantify individual charge variants or acidic and basic totals

Hydrophilic Interaction Chromatography (HILIC) Separations



Hydrophilic Interaction Chromatography (HILIC) Mechanism

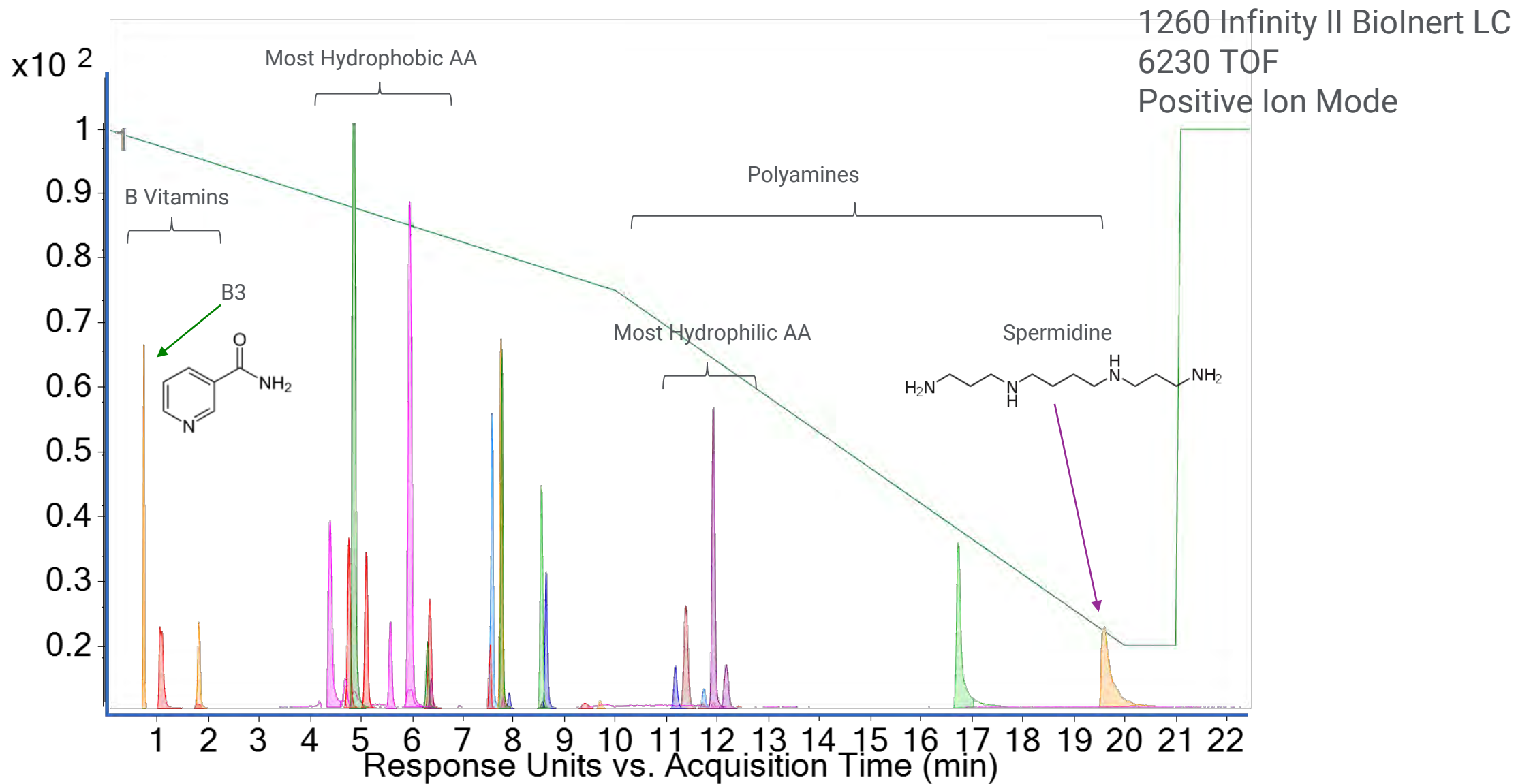
- Polar analytes are retained by polar or ionic interaction with polar stationary phase and H₂O layer surrounding stationary phase.
- Analytes are eluted by increasing H₂O concentration in mobile phase
- Reverse of reversed phase
 - Uses same mobile phase components as reversed phase with an opposite gradient (H₂O increased rather than decreased)
 - The distinction between normal phase is that HILIC uses H₂O in mobile phase whereas normal phase uses organic mobile phase components



HILIC Advantages and Applications

- Retains small polar molecules and charged molecules that cannot be sufficiently retained on reversed phase columns
- Applications include: glycan analysis, underivatized amino acids and other components of growth media
- Very mass spectrometry friendly

Survey a Wide Range of Spent Media Metabolites

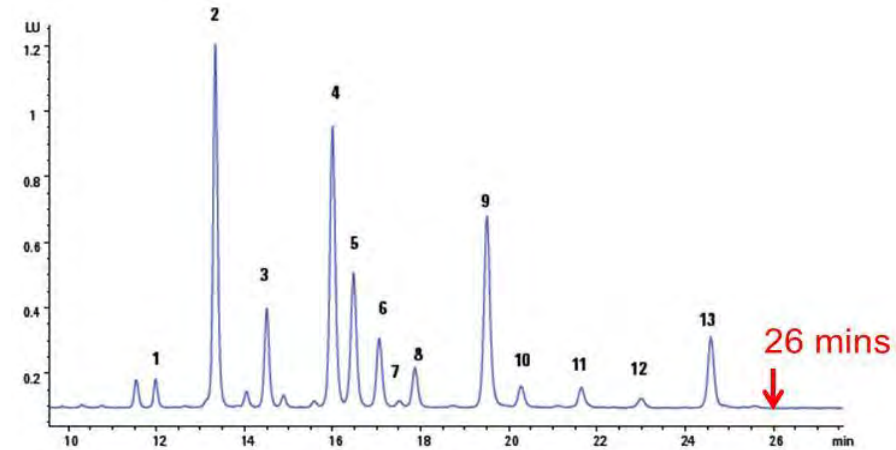


Released N-Glycan Analysis Using HILIC Amide Columns



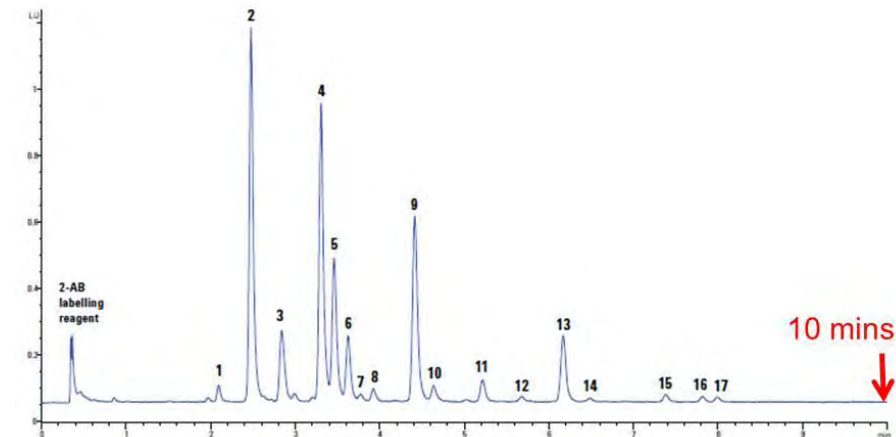
+

AdvanceBio
Glycan Mapping,
2.7 μm

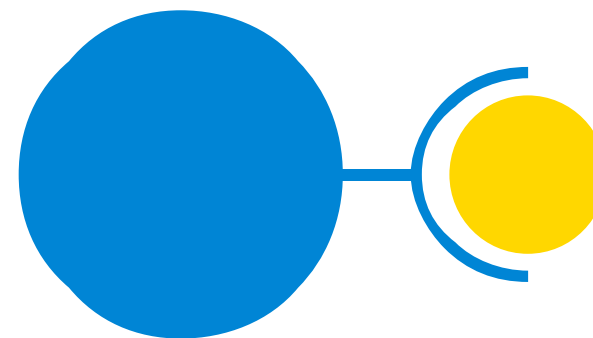


+

AdvanceBio
Glycan Mapping,
1.8 μm

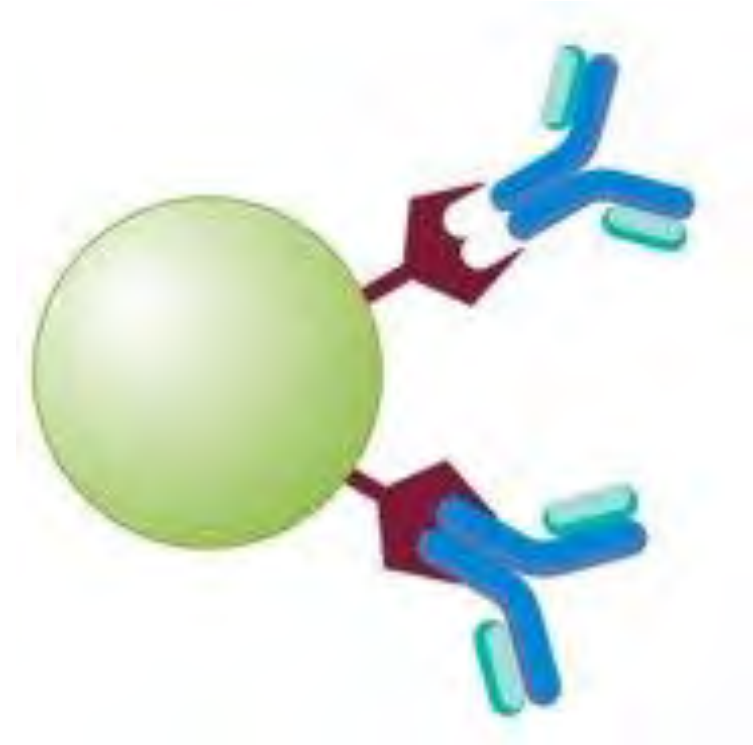


Affinity Separations



Affinity Chromatography Mechanism

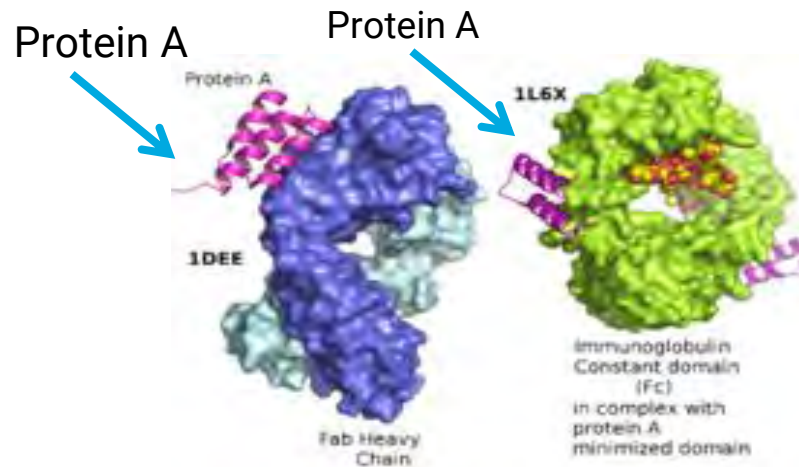
- Stationary phase includes bound ligands with a specific affinity for molecule of interest
- Typically used to separate a molecule of interest from a complex matrix
- Commonly used for prep/purification
 - Bind molecule of interest while flow through goes to waste
 - Elute to collect
- Protein A has a specific affinity for antibodies
 - Used for prep workflows and to purify small amounts of antibody for titer determination or other analysis



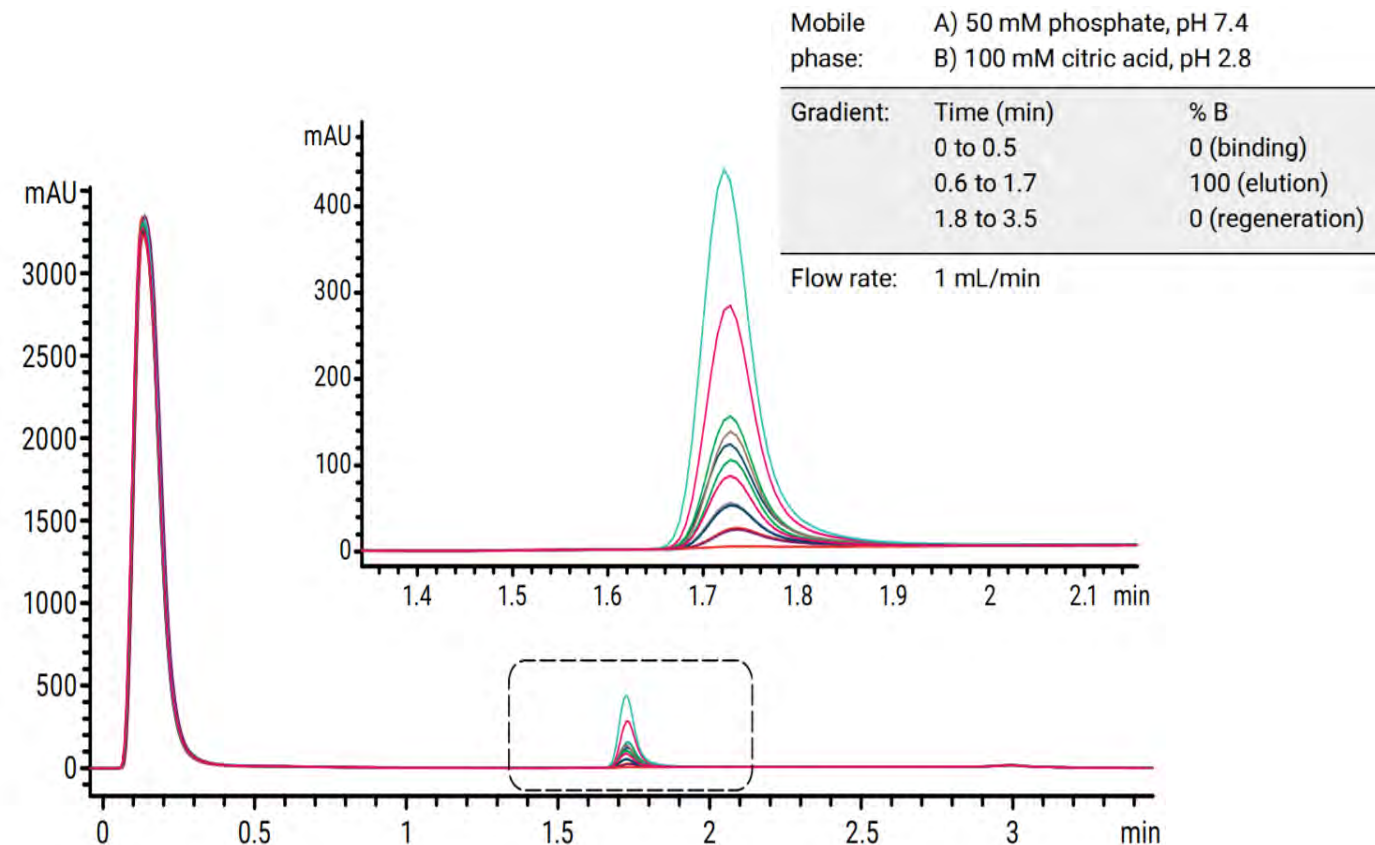
Titer Analysis - Agilent BioMonolith Protein A Column

- Fast screening of harvest cell culture samples for IgG – process optimization
- Accurate analysis of mAb quantities to determine protein harvest
- Capture and purification of protein for further characterization
- Monolith material does not clog easily with cell debris

Overlaid UV 280 nm Bio-Monolith Protein A chromatograms of 12 trastuzumab-producing CHO clones:



http://en.wikipedia.org/wiki/Protein_A



Binding Affinities of Protein A and Protein G

Antibody	Antibody	Protein A	Protein G
Human	Human IgG1	++++	++++
	Human IgG2	++++	++++
	Human IgG3	-	++++
	Human IgG4	++++	++++
	Human IgA	++	-
	Human IgD	++	-
	Human IgE	++	-
	Human IgM	++	-
Mouse			
	Mouse IgG1	+	++
	Mouse IgG2a	++++	++++
	Mouse IgG2b	+++	+++
	Mouse IgG3	++	+++
	Mouse IgM	+/-	-
Rat			
	Rat IgG1	-	+
	Rat IgG2a	-	++++
	Rat IgG2b	-	++
	Rat IgG2c	+	++
	Rat IgM	+/-	-
	Rabbit IgG	++++	+++
Others			
	Hamster IgG	+	++
	Guinea Pig IgG	++++	++
	Bovine IgG	++	++++
	Sheep IgG	+/-	++
	Goat IgG	+/-	++
	Pig IgG	+++	+++
	Chicken IgG	-	+

Antibody	Protein A	Protein G
<i>Fragments</i>		
Human Fab	+	+
Human F(ab') ₂	+	+
Human scFv	+	-
Human Fc	++	++
Human K	-	-
Human λ	-	-

Key code for relative affinity of Protein A & G for respective antibodies:

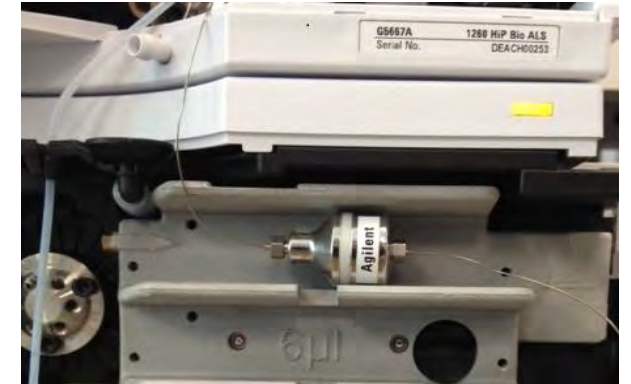
++++ = Strong affinity

+++ = Moderate affinity

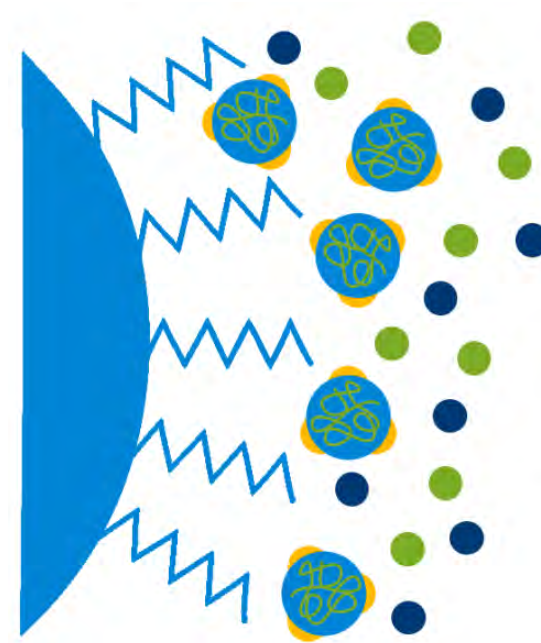
++ = Weak affinity

+ = Slight affinity

- = No affinity

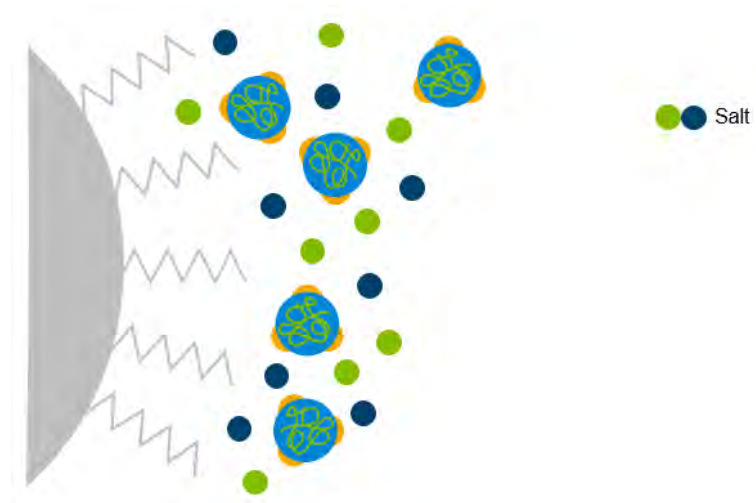


Hydrophobic Interaction Chromatography (HIC) Separations

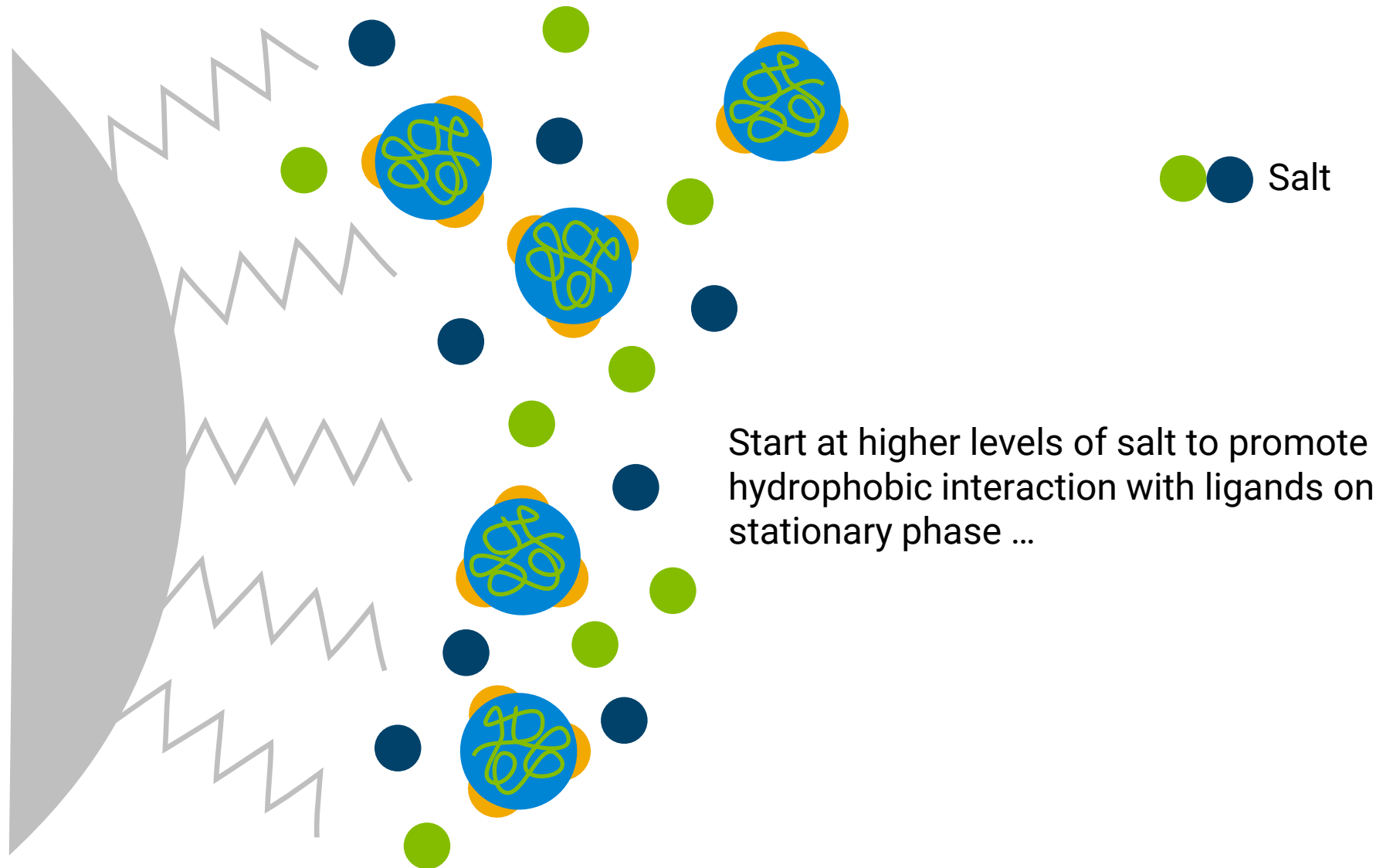


Hydrophobic Interaction Chromatography (HIC) - Mechanism

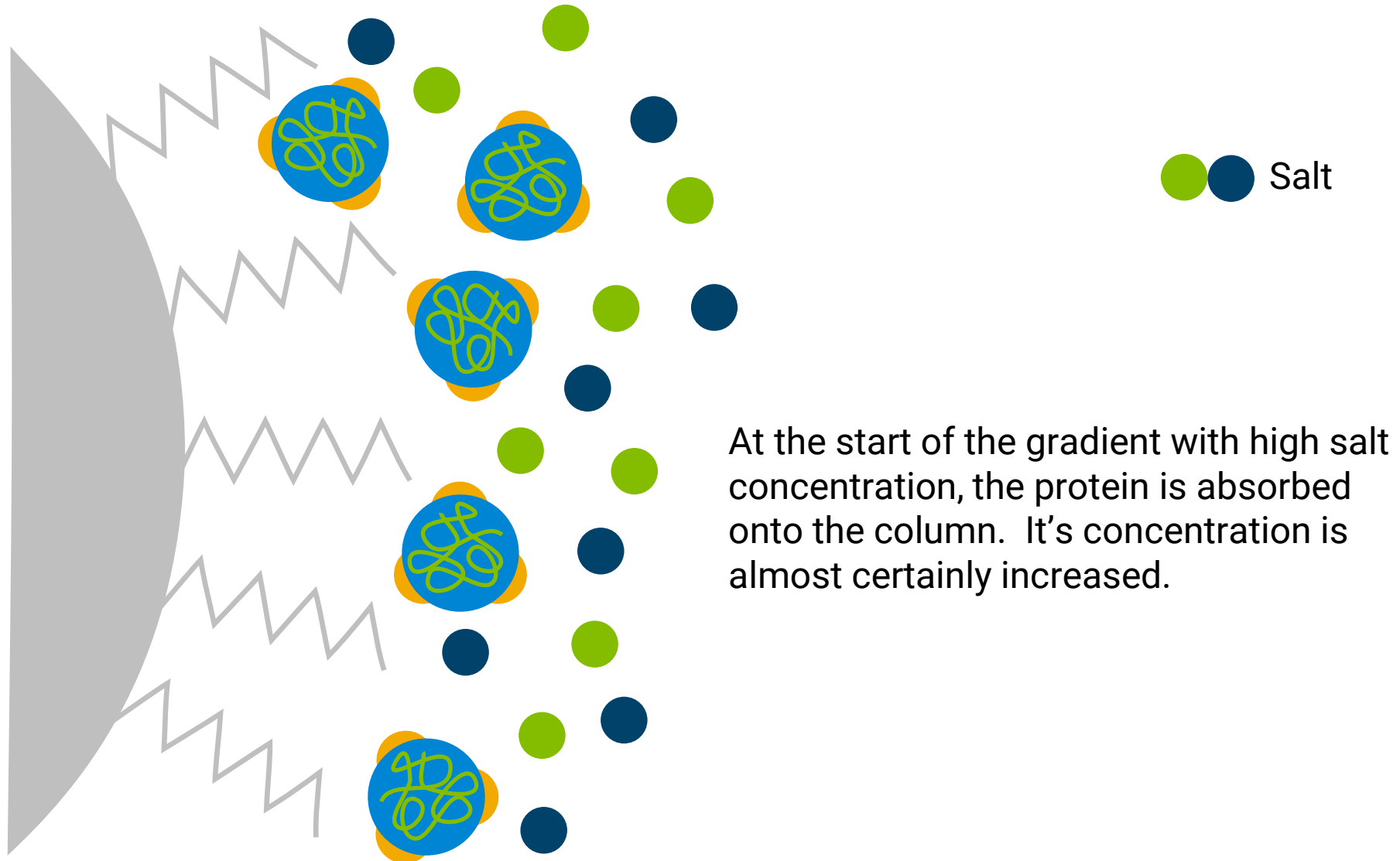
- Analytes are retained on a mildly hydrophobic stationary phase (much less hydrophobic than in Reversed Phase)
- Retention on the stationary phase is driven by high salt concentrations in the aqueous mobile phase.
- Analytes elute in order of increasing hydrophobicity by a gradient starting in high salt and going to low salt



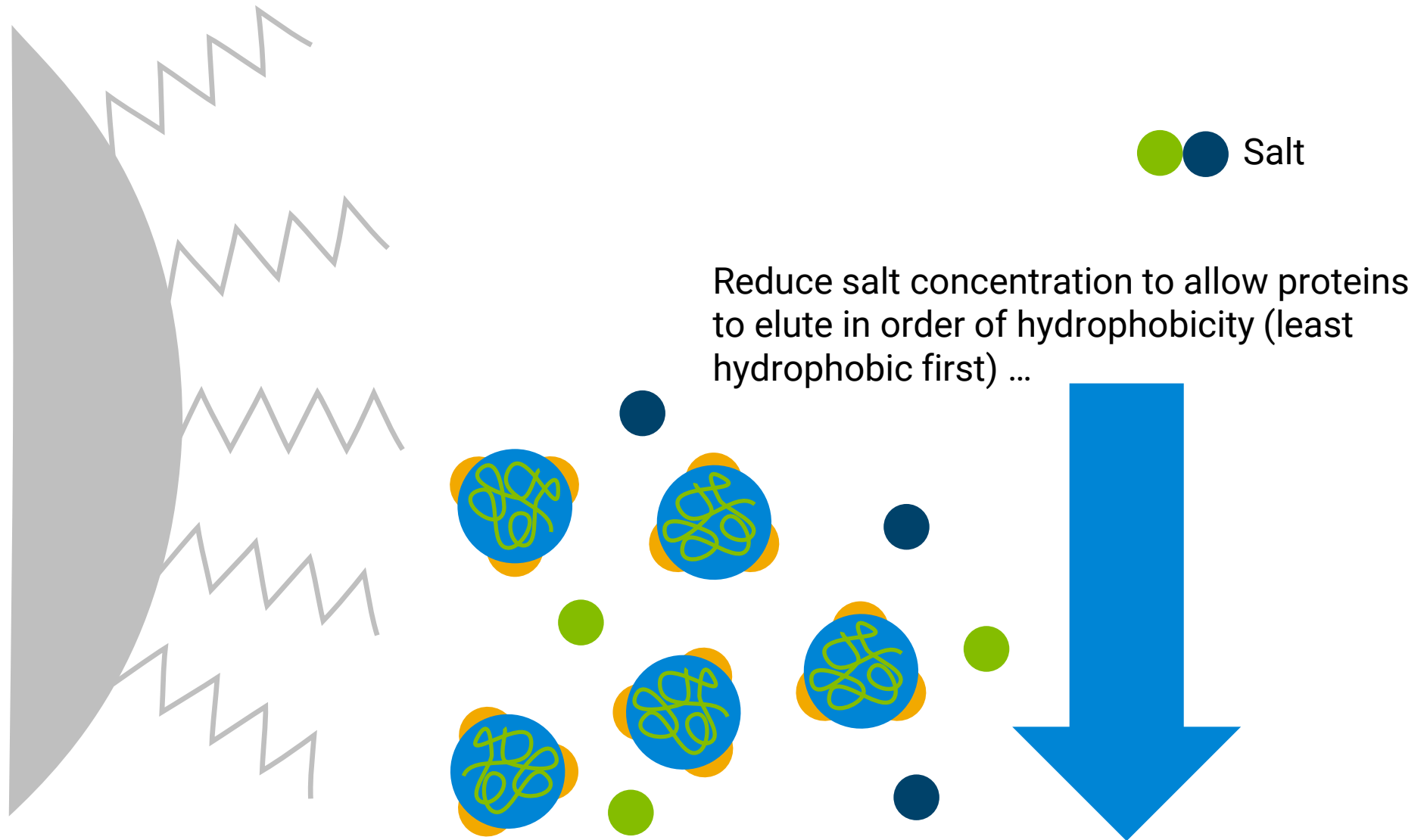
Protein Adsorption on HIC Stationary Phase



Protein Adsorption on HIC Stationary Phase



Protein Elution on HIC Stationary Phase



Hydrophobic Interaction Chromatography (HIC)

Advantages and Applications



- HIC allows **native state** analysis of proteins in whereas RPLC is denaturing
 - Aqueous mobile phase and lower temperatures maintains protein folding
- Historically used for purification as it is non-denaturing but gaining use as an analytical technique
- Proteins can be collected in their native state for further functional assays such as cell based potency, receptor binding, cell proliferation assay, functional ELISA

Hydrophobic Interaction Chromatography (HIC)

Advantages and Applications

- **Post translational modifications:** Alternative selectivity to other chromatographic approaches
 - oxidised species, deamidation, isoaspartate formation
 - subtle changes that expose more, or less, of a hydrophobic surface.
- **Antibody Drug Conjugates (ADCs) Drug Antibody Ratio (DAR) analysis:**
 - Maintains intact confirmation of cysteine linked ADCs
- **Bispecific antibody heterodimerization efficiency:**
 - If parental mAbs have different hydrophobicity the bispecific peak elutes between peaks of residual parental mAbs.
- **Hydrophobicity determination (in native state):**
 - Used for predicting physicochemical characteristics of a biotherapeutic candidate

Conclusion

- Understanding separation principles simplifies method development, troubleshooting, and predicting behavior
- Biopharmaceuticals are complex molecules requiring many chromatography techniques to characterize
- Each chromatography mode has advantages for particular applications