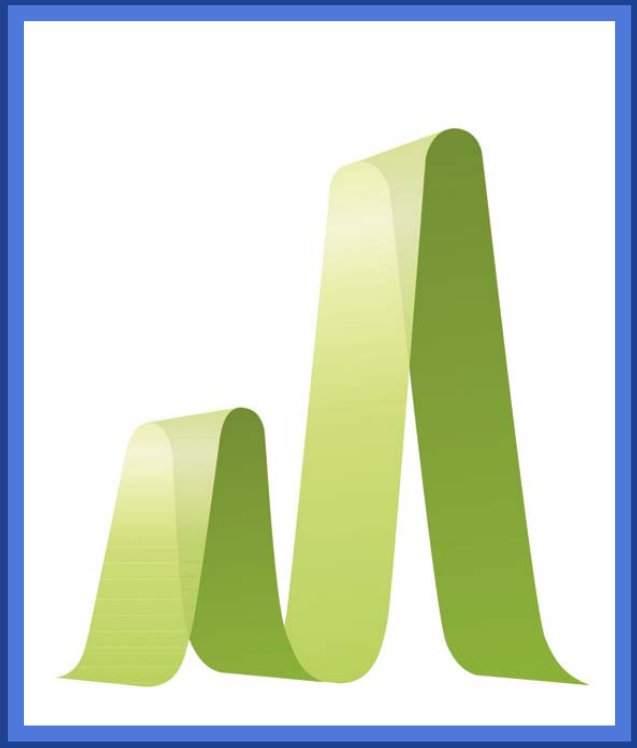
Gel filtration
凝胶过滤层析技术



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Content 内容

Principles of gel filtration 凝胶过滤原理

Optimize : How to get the expected results 优化

Products and applications 产品和应用

Practical considerations 实际需考虑的因素

Summary 总结

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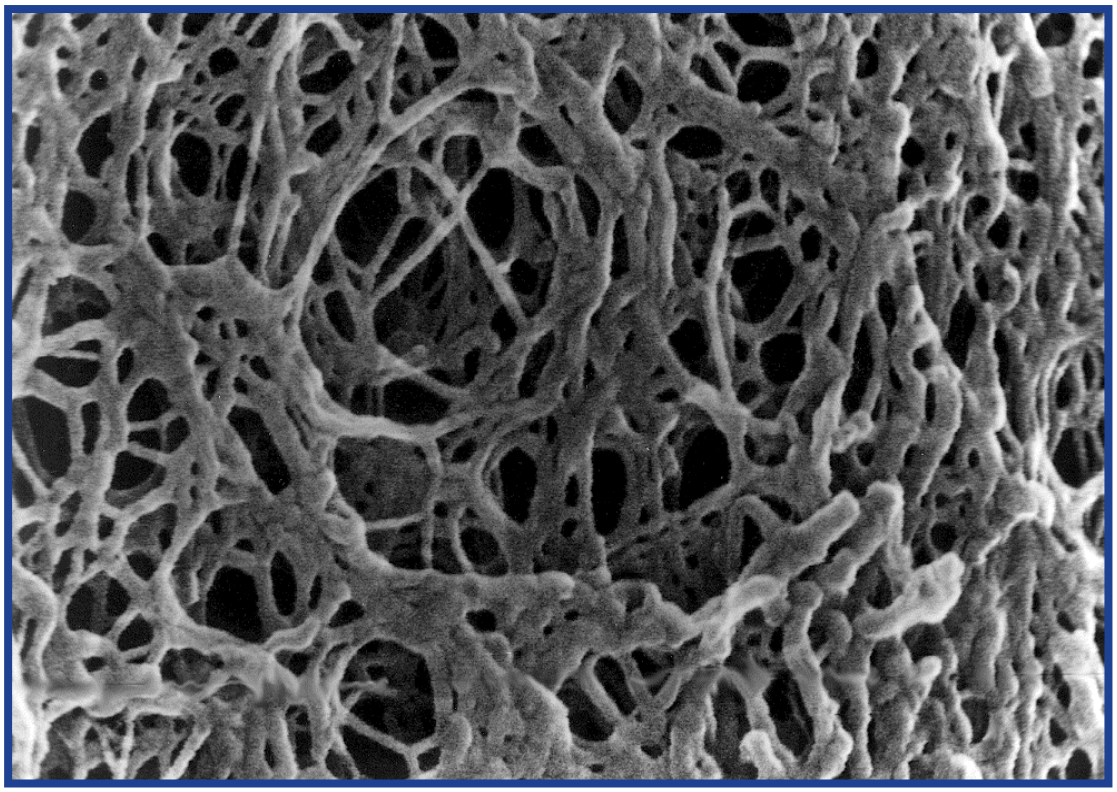
What is gel filtration凝胶过滤定义?

Gel filtration (GF) is a simple and reliable chromatographic method for separating molecules according to size and shape.

凝胶过滤层析是根据分子大小和形状的差异进行分离的一种简单可靠的层析技术。

Gel structure 填料结构

AGAROSE 琼脂糖凝胶



A good gel for gel filtration contains about
95% water

凝胶过滤填料的
95%为水

- pores filled with eluent 孔内充满洗脱液
- carefully controlled size range 孔径精密控制
- chemically /physically inert (lack of adsorptive properties) 惰性

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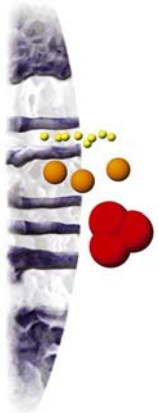
凝胶过滤分离原理：空间排阻

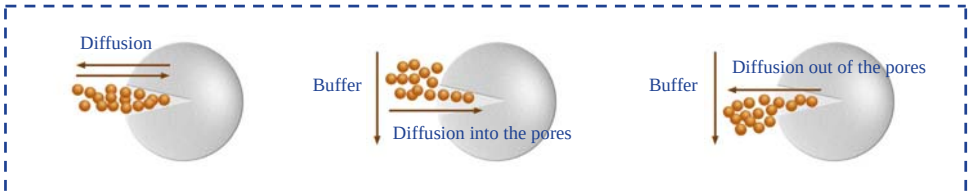
1. Spherical particles packed into a column.

2. Sample applied.



3. Buffer (mobile phase) and sample move through column. Molecules diffuse in and out of matrix

4. Large molecules leave the column first followed by smaller molecules in order of size. Molecules larger than the matrix pores pass straight through.



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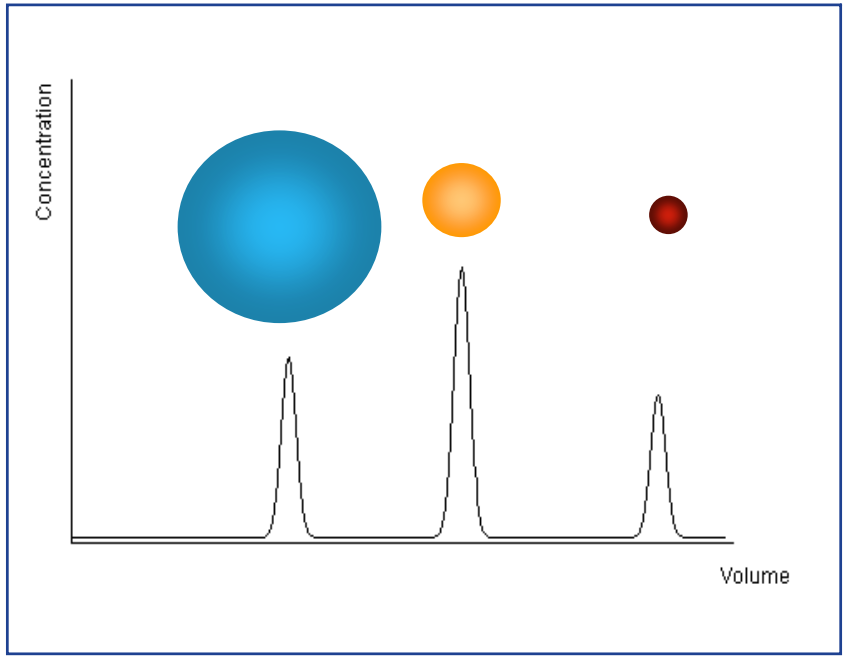
Steric exclusion leads to early elution
体积排阻程度决定洗脱位置

Molecules elute in order of size.

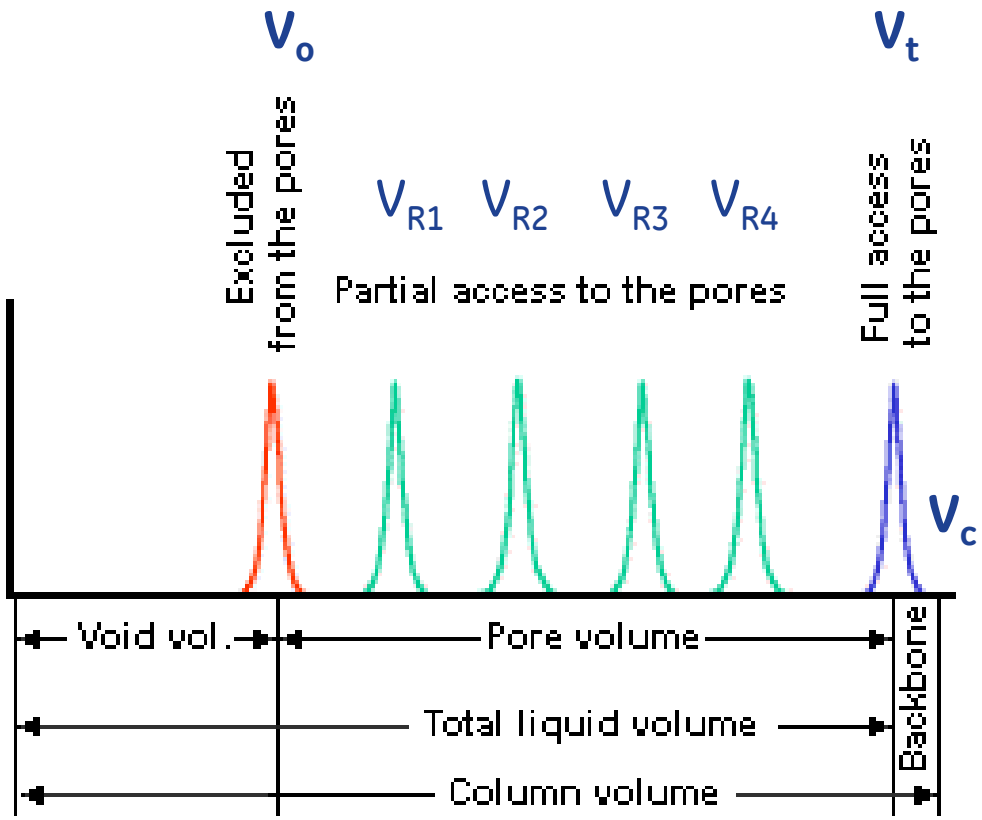
不同分子按照大小顺序洗出

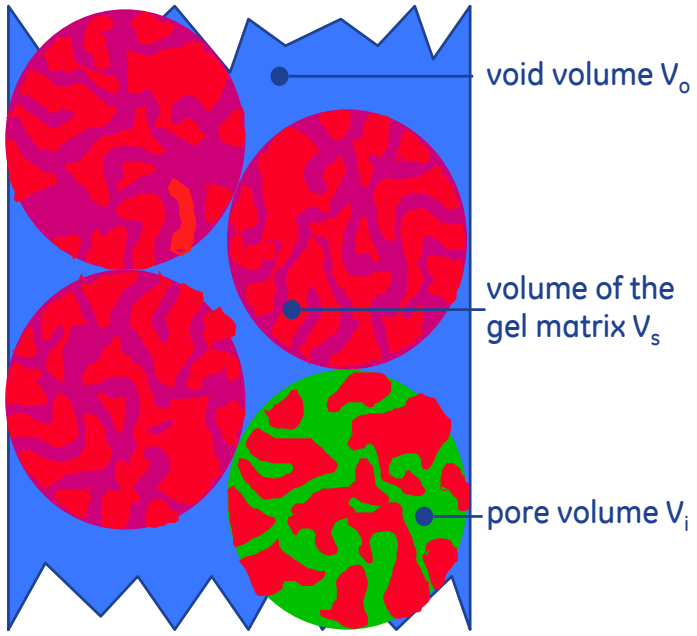
The largest molecules elute first; the smallest ones elute last.

大分子先洗出, 小分子后洗出



Separation volumes 分离体积





V_o = void volume 外水体积

V_R = retention volume or elution volume 保留体积/洗脱体积

V_t = total liquid volume of the bed 总液体体积

V_i = inner pore volume = $V_c - V_s - V_o$ 内孔体积/内水体积

V_c = total geometric volume of the column 柱体积

V_s = volume of gel matrix 固相凝胶的体积

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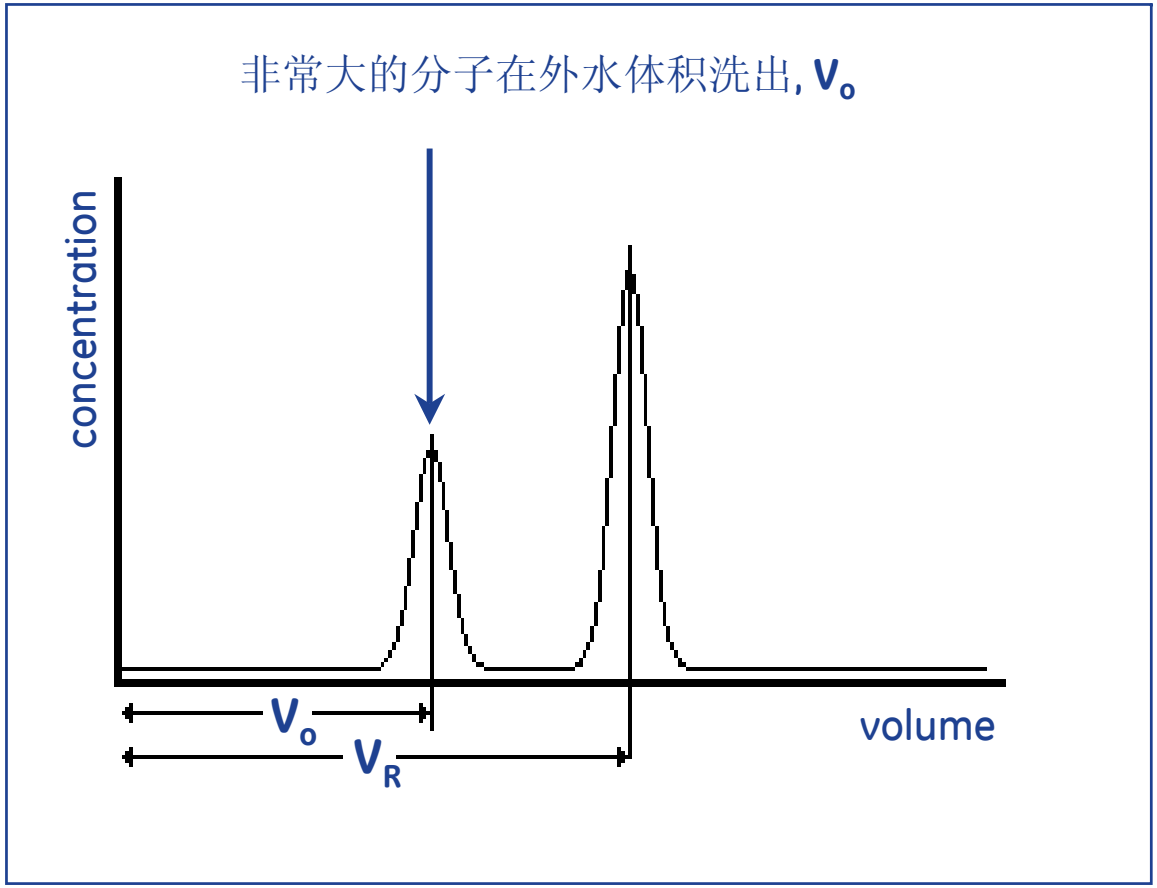
The void volume V_o 外水体积



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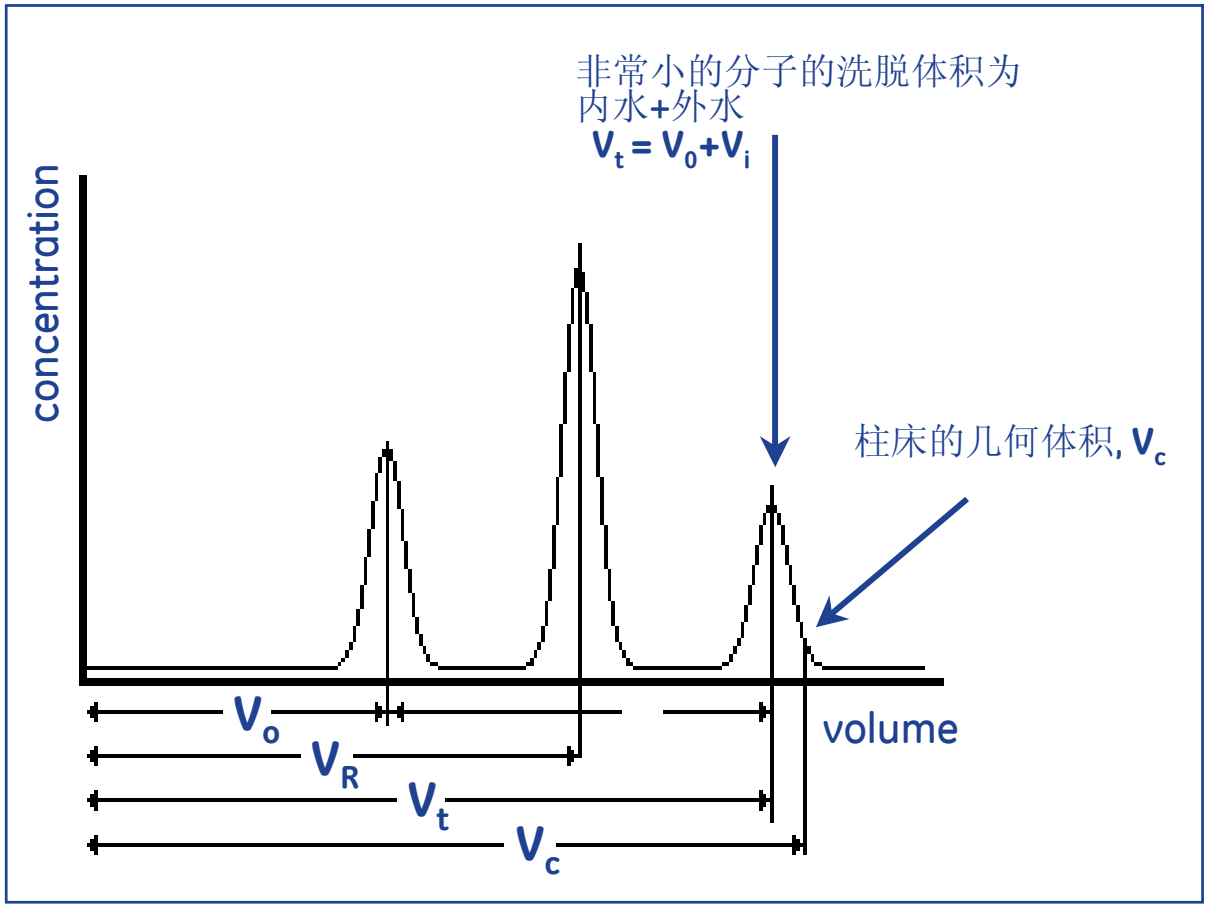
V_t and V_c 总液体体积和柱体积



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Distribution coefficient, K_d 分配系数

$$K_d = \frac{V_R - V_0}{V_c - V_0 - V_s} = \frac{V_R - V_0}{V_i}$$

i.e. , $V_R = V_o + K_d * V_i$

V_R = 保留体积/洗脱体积

V_o = 排阻体积/外水体积

V_c = 层析柱几何体积

V_s = 固相凝胶所占的体积

V_i = 孔内体积/内水体积 = $V_c - V_s - V_o$

-V_R : 外水体积 + 能进入的那部分内水体积

-K_d : 溶质分子能够进入的内水体积 (内水体积)占总的内水体积的比例

K_d 较难测定, 常用Kav代替

The coefficient K_{av}

$$K_{av} = \frac{V_R - V_0}{V_c - V_0}$$

- 与柱子大小和填装无关
- 可以用于比较不同的纯化结果

K_{av} 容易得到，也更有实际意义

K_{av} for very large and very small molecules

非常大的分子在 V_0 处洗脱

$$V_R = V_0$$

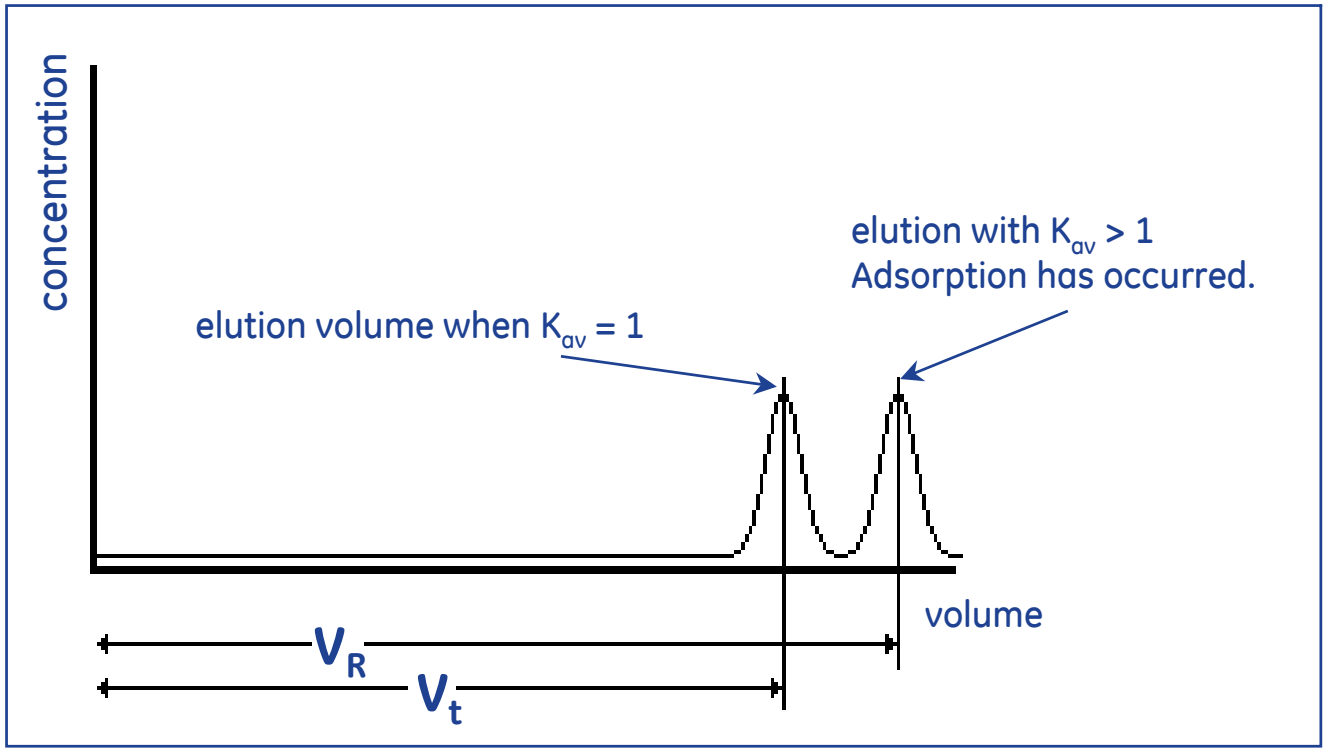
$$K_{av} = \frac{V_R - V_0}{V_c - V_0} = \frac{V_0 - V_0}{V_c - V_0} = 0$$

非常小的分子在 V_t 处洗脱

$$V_R = V_t$$

$$K_{av} = \frac{V_R - V_0}{V_c - V_0} = \frac{V_R - V_0}{V_R - V_0} = 1$$

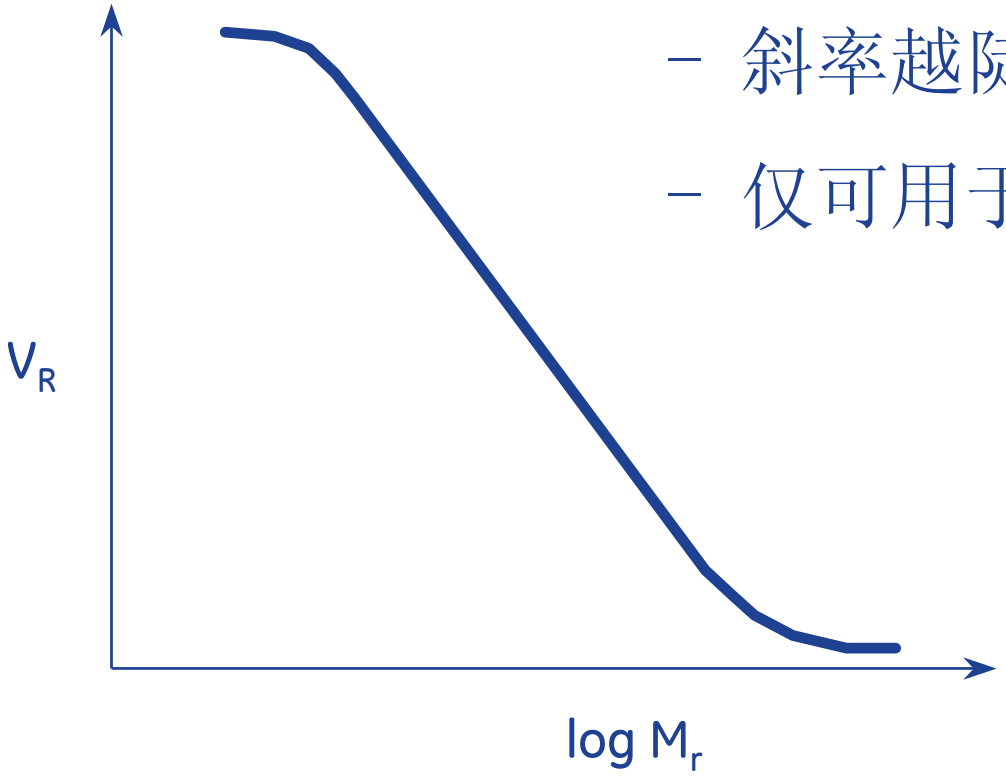
K_{av} 应该在0和1之间



elution with K_{av} < 0
Serious problem!

流动相中需要加入150mM NaCl！

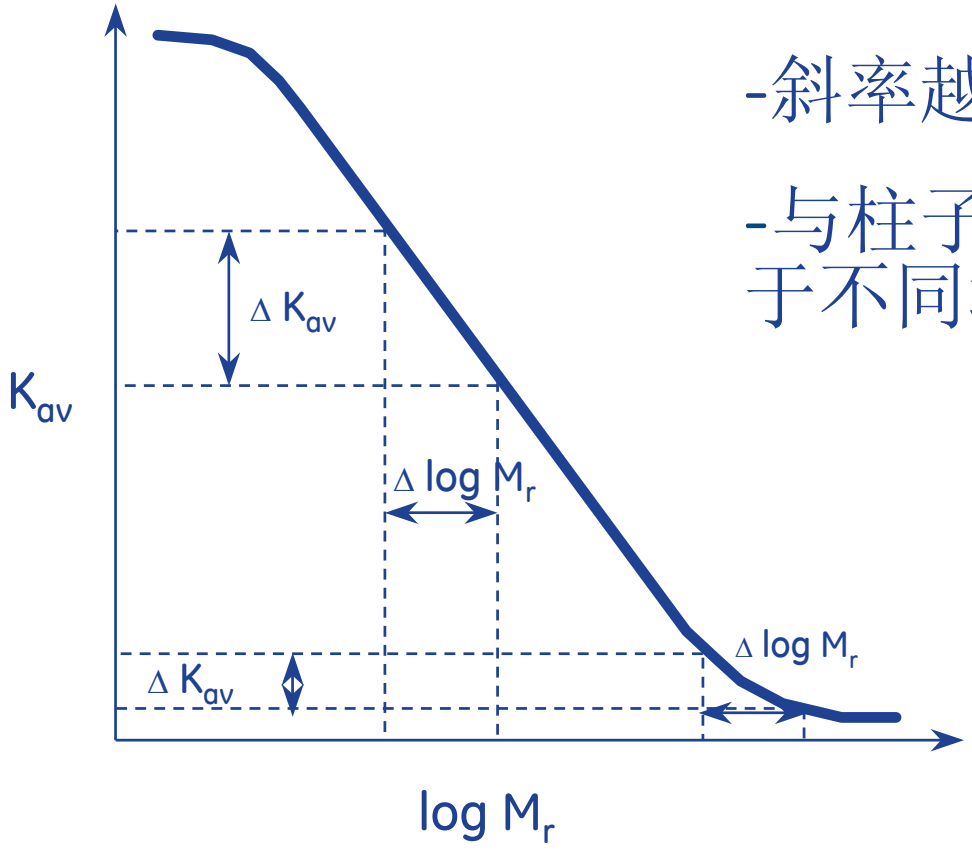
Calibration curves 校准曲线



- 斜率越陡，洗脱体积差异越大
- 仅可用于特定的柱子

V_R *versus* the logarithm of molecular mass

Selectivity curves 选择性曲线



K_{av} *versus* **the logarithm of molecular mass**

-斜率越陡, 洗脱体积差异越大

-与柱子的填装无关，可以用于不同填料的性质比较

$$K_{av} = \frac{V_R - V_o}{V_c - V_o}$$

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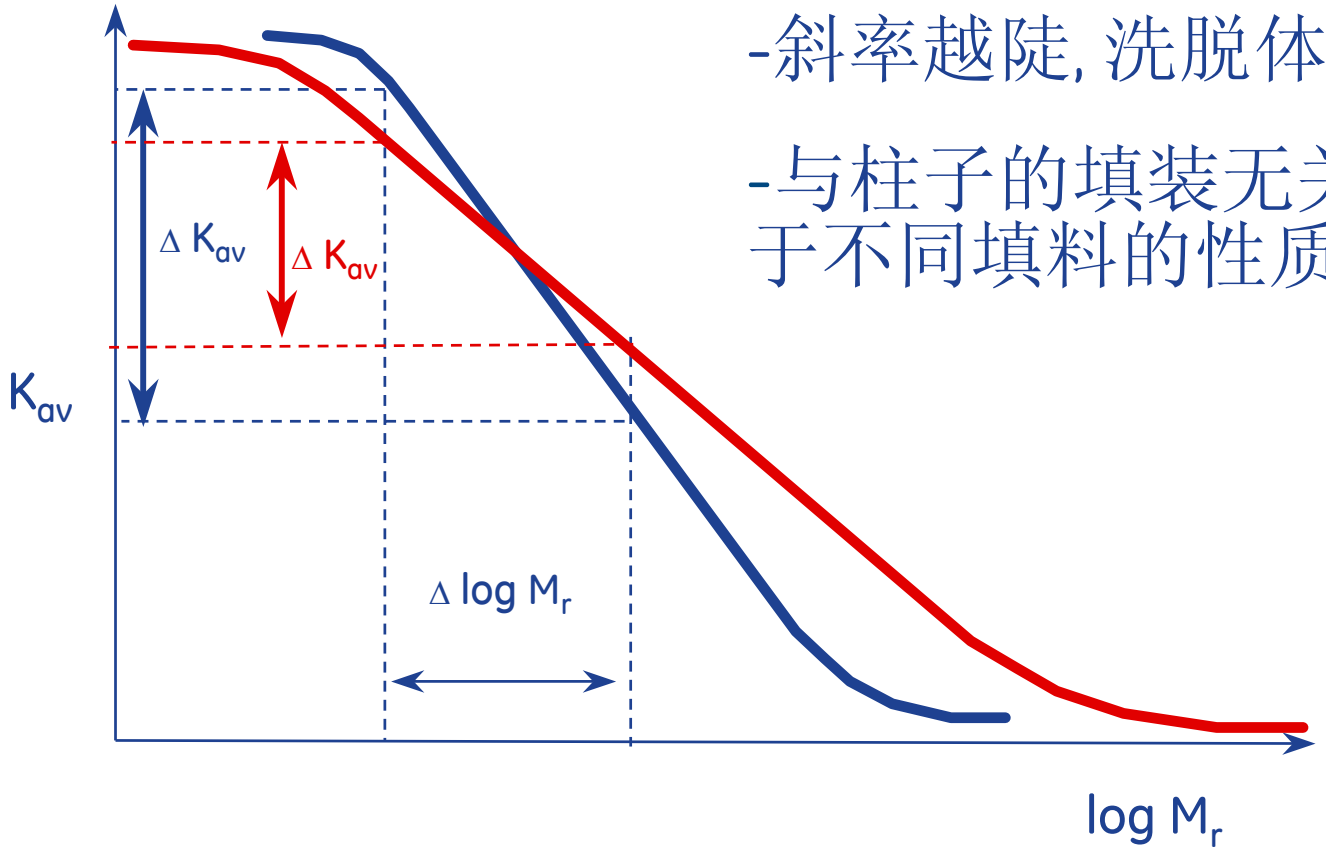
Selectivity curves 选择性曲线



-斜率越陡, 洗脱体积差异越大

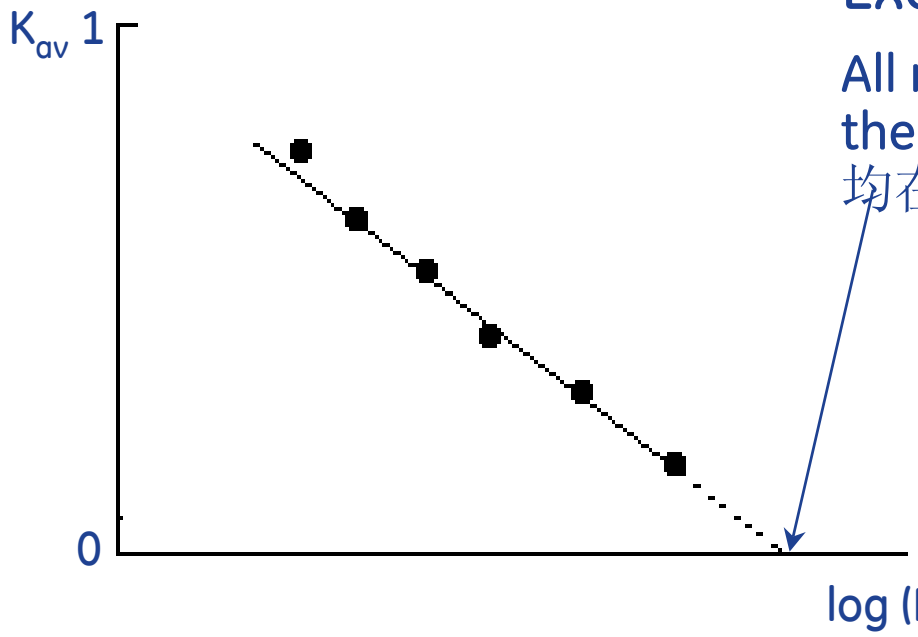
-与柱子的填装无关，可以用于不同填料的性质比较

$$K_{av} = \frac{V_R - V_o}{V_c - V_o}$$

K_{av} *versus* the logarithm of molecular mass

Exclusion limit 排阻极限

不能进入填料孔的最小的分子量

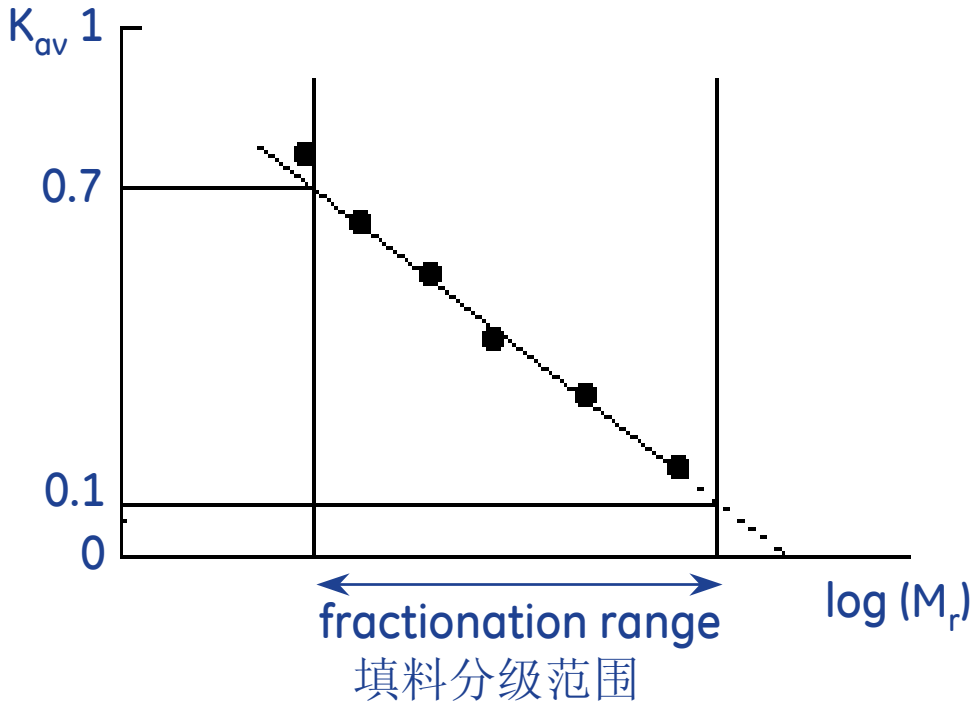


Exclusion limit

All molecules bigger than this elute in the void volume.分子量大于此的分子均在外水体积洗出

Fractionation range 分级范围

K_{av}=0.1 to K_{av}=0.7的范围内，选择性曲线为直线



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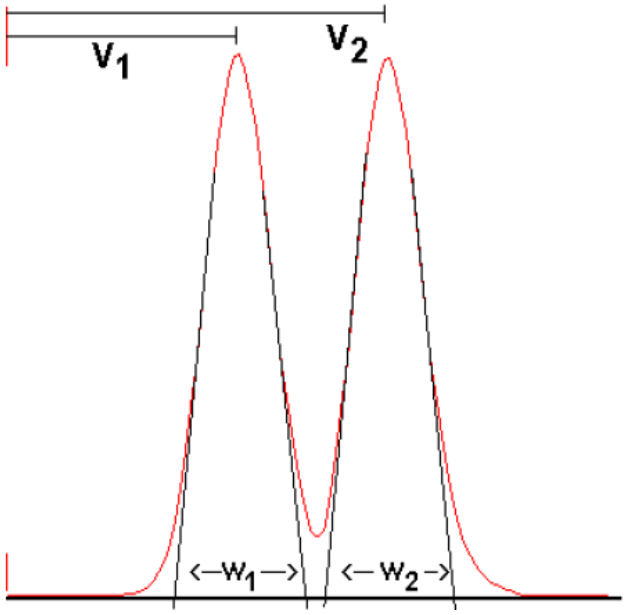
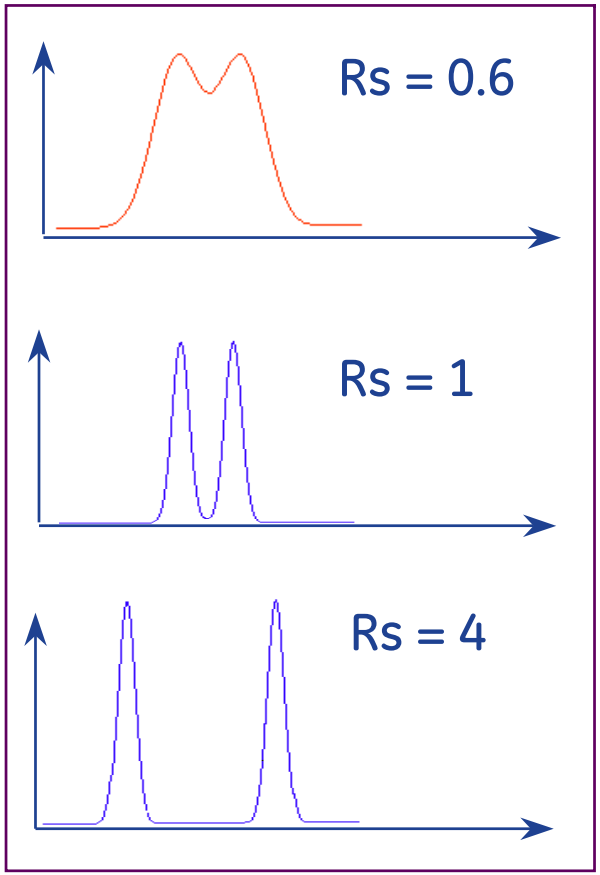
Resolution factor, R_s 分辨率

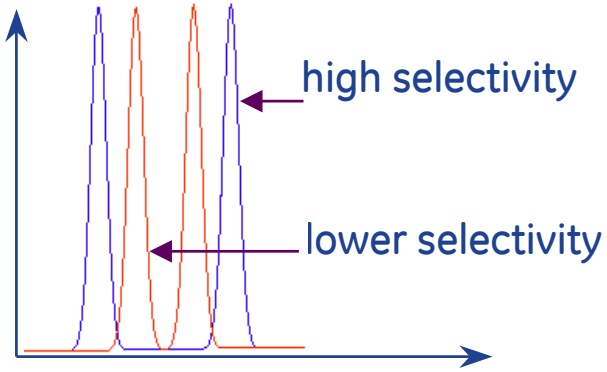


$$R_s = \frac{V_2 - V_1}{(W_1 + W_2) / 2}$$



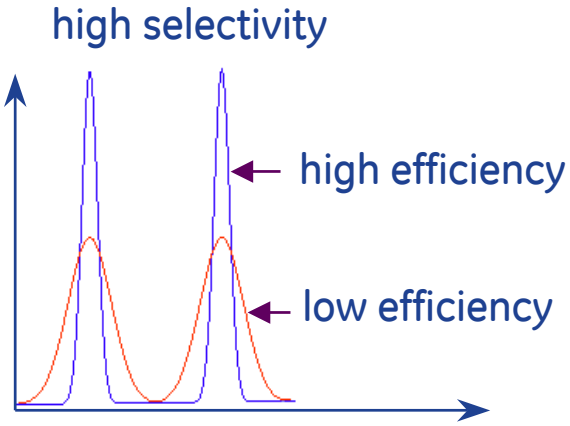
两峰相对于其峰宽而言，分开的有多远

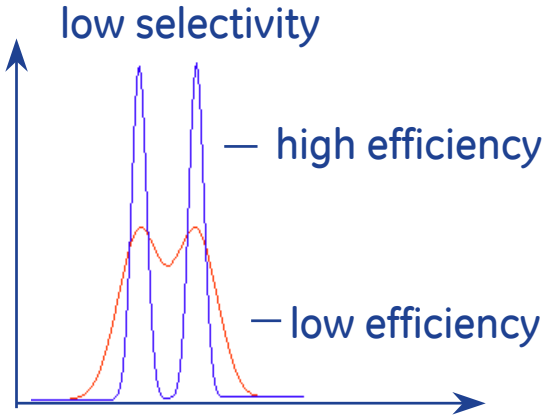
Resolution: depends on 分辨率取决于 selectivity 选择性 and efficiency 柱效



Selectivity 选择性:

- 峰分离的量度
- 高选择性可以达到基线分离
- 高选择性可以弥补低的柱效
  - 峰体积有可能增加





Efficiency 柱效:

- 峰宽的量度
- 柱效越高峰越窄
- 高柱效需要良好的柱填装技术
- 高柱效有时可弥补选择性不足

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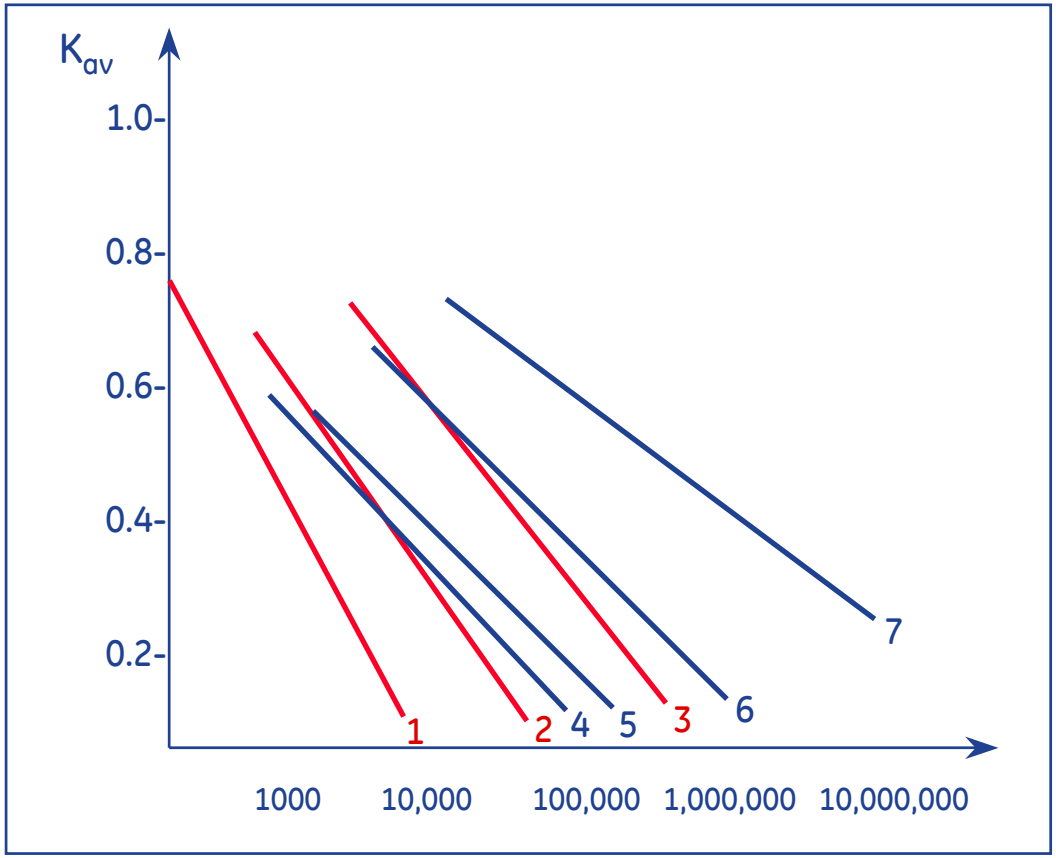
选择最佳的凝胶 (high selectivity)



1. Superdex™ Peptide
2. Superdex 75
3. Superdex 200
4. Sephacryl™ S-100 HR
5. Sephacryl S-200 HR
6. Sephacryl S-300 HR
7. Sephacryl S-400 HR

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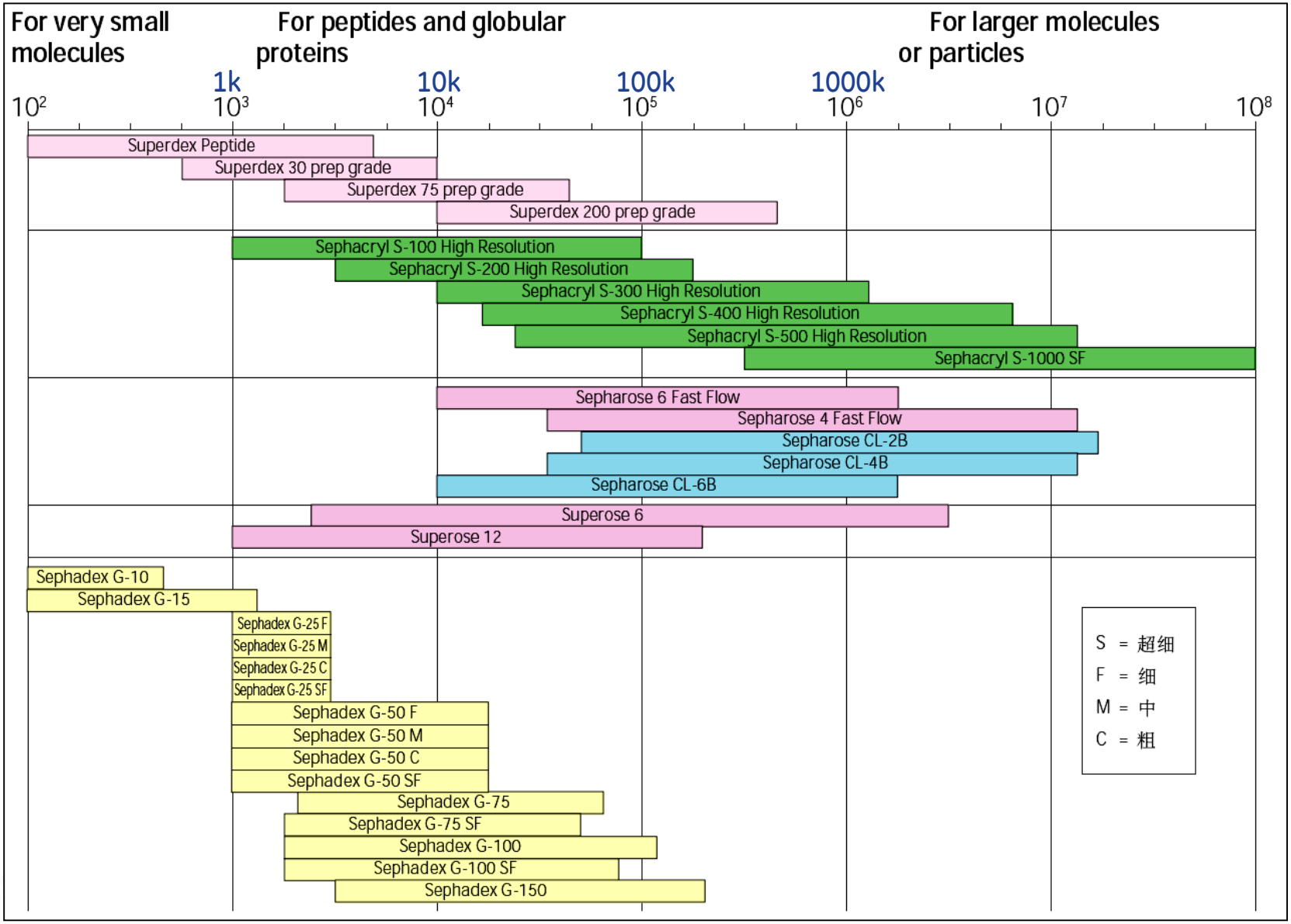
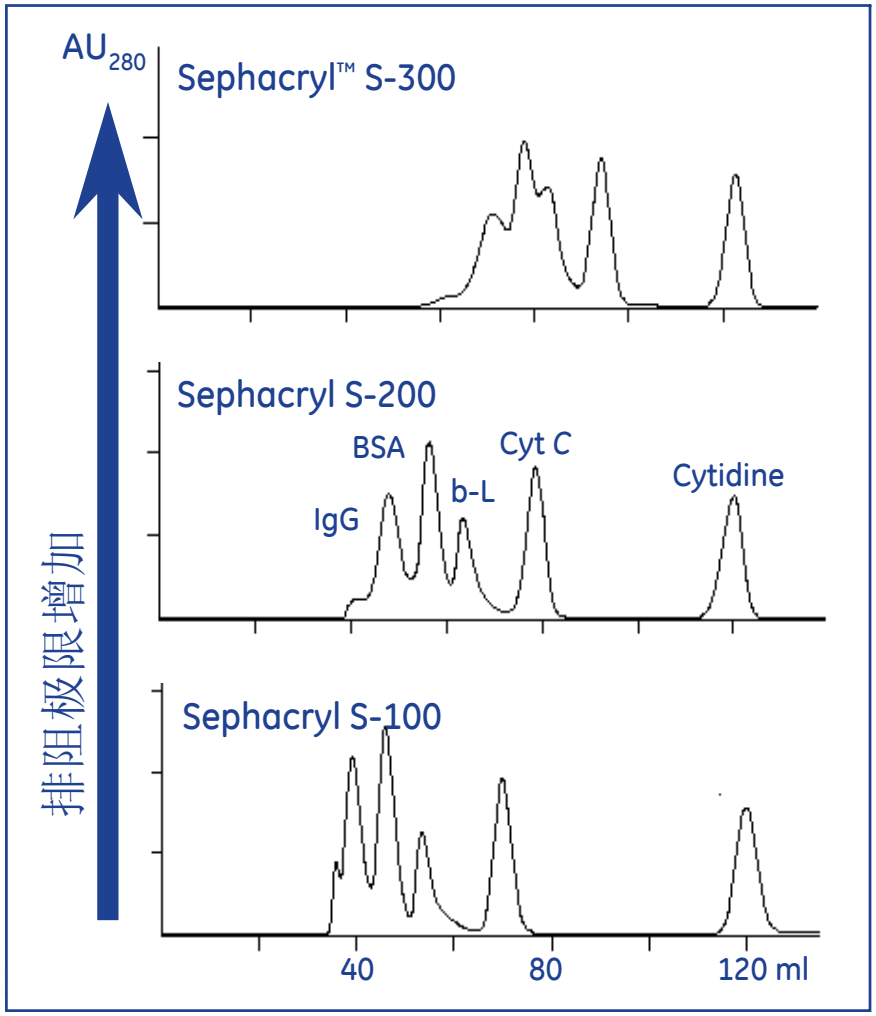
Fractionation Range 分级范围



Results depend on selectivity 分离结果与选择性有关

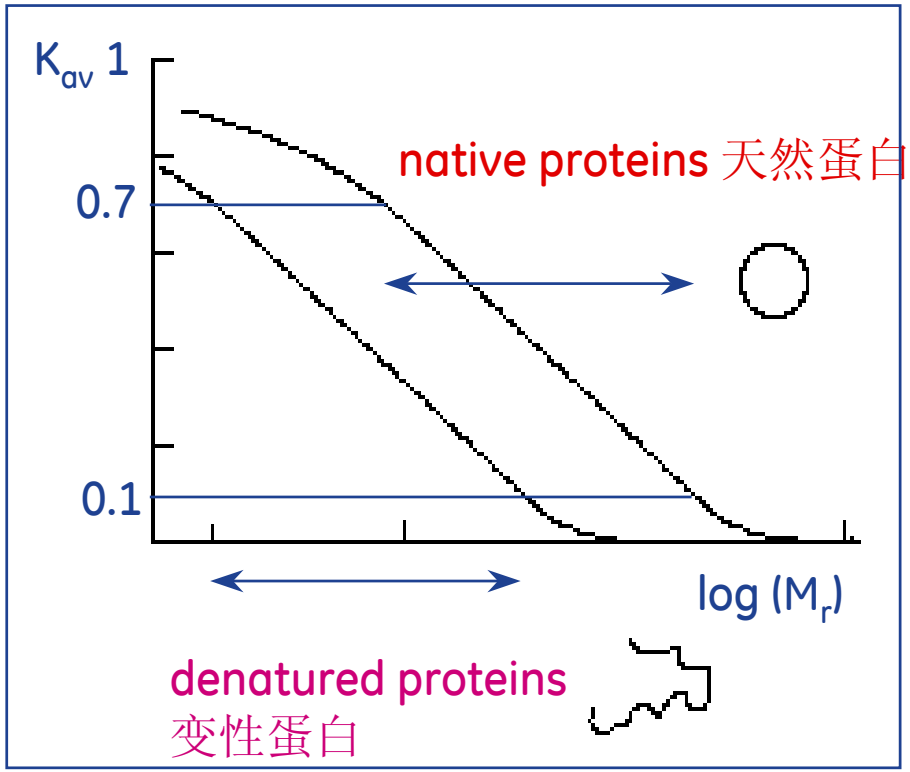


10k-1500k
适合较大分子

5k-250k
最佳

1k-100k
适合较小分子

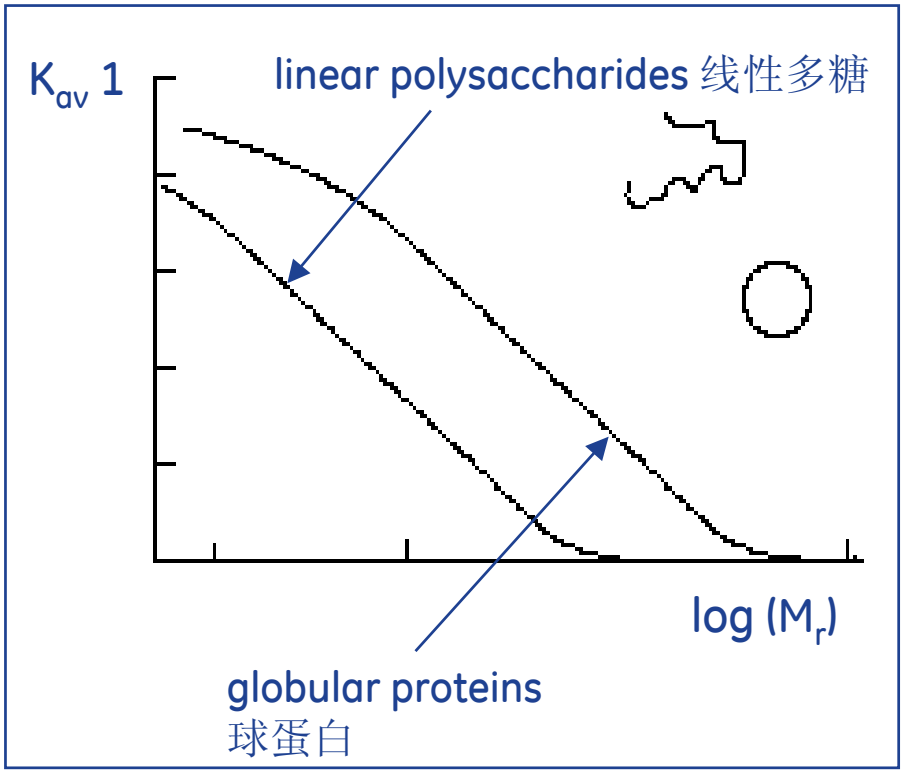
Shape effects 形状的影响



A gel has different fractionation ranges for native, globular proteins and denatured proteins in random coil.

对于形状不同的分子，某填料的分级范围不同

Shape effects 形状的影响



Molecules with different shapes have different selectivity curves.

对于形状不同的分子，有不同的选择性曲线。

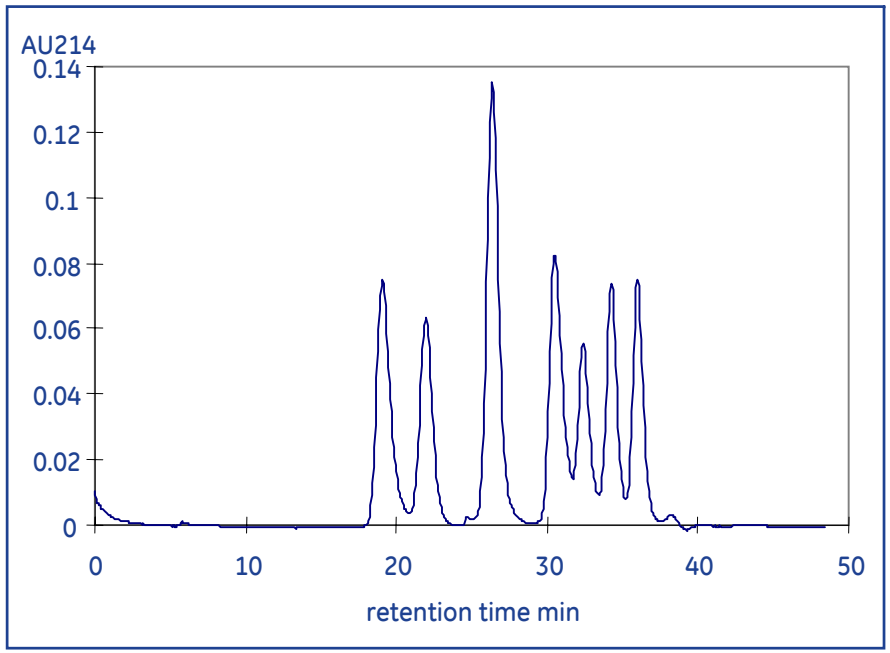
Efficiency 柱效

Efficiency depends on 取决于：

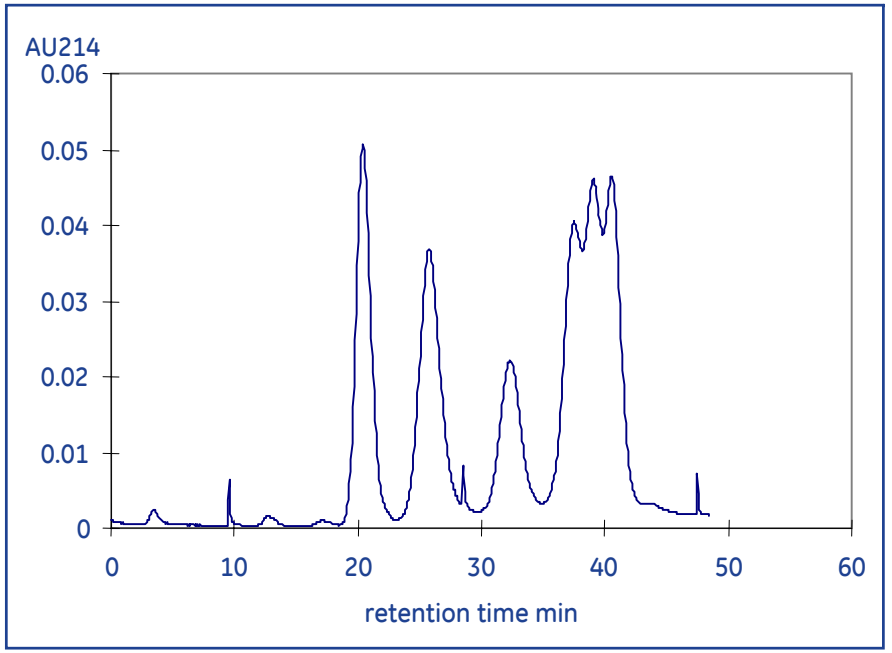
- **flow rate** 流速
- particle size of matrix 粒径大小
- particle size distribution of matrix 粒径分布
- packing quality of the column 层析柱的装填
- sample volume and viscosity 样品体积和黏度

Peak width depends on particle size
粒径影响柱效

Superdex™ Peptide 13-15 µm



Superdex 30 prep grade 24-44 µm



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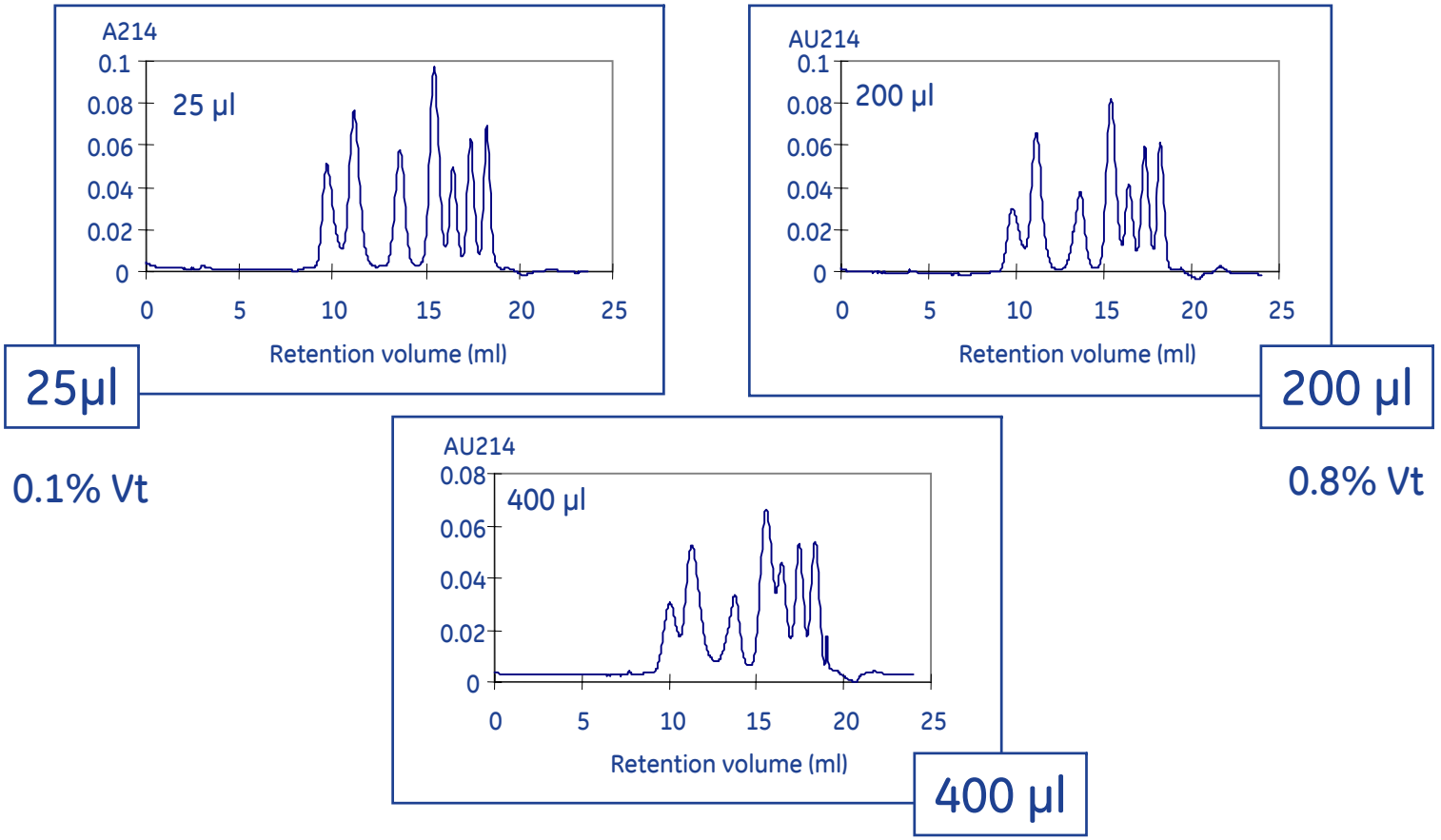
Resolution depends on sample volume
进样体积影响柱效

Superdex™ Peptide High Performance column 10/30_24ml



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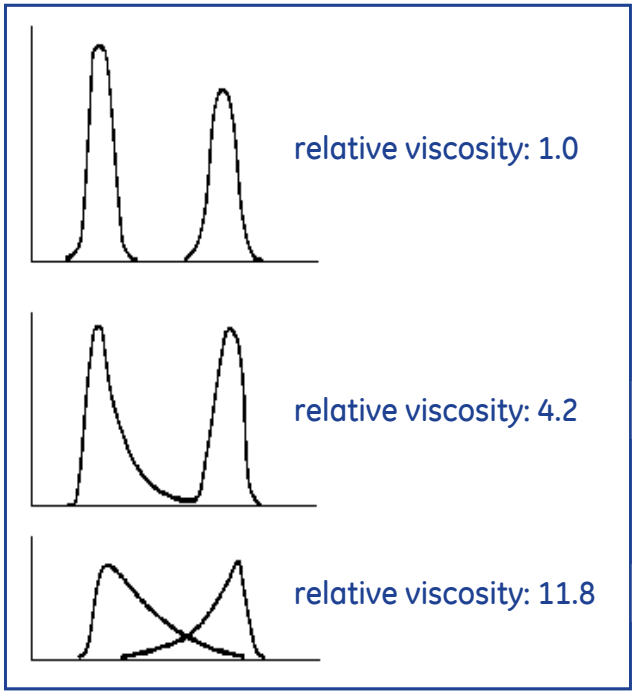
Resolution depends on sample viscosity 黏度影响柱效

样品黏度过高，分离度下降.

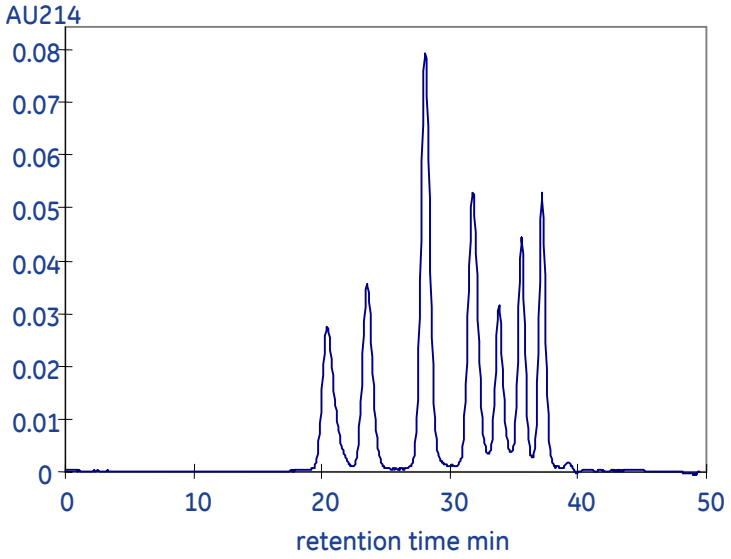


Haemoglobin and NaCl, viscosity changed by adding dextran

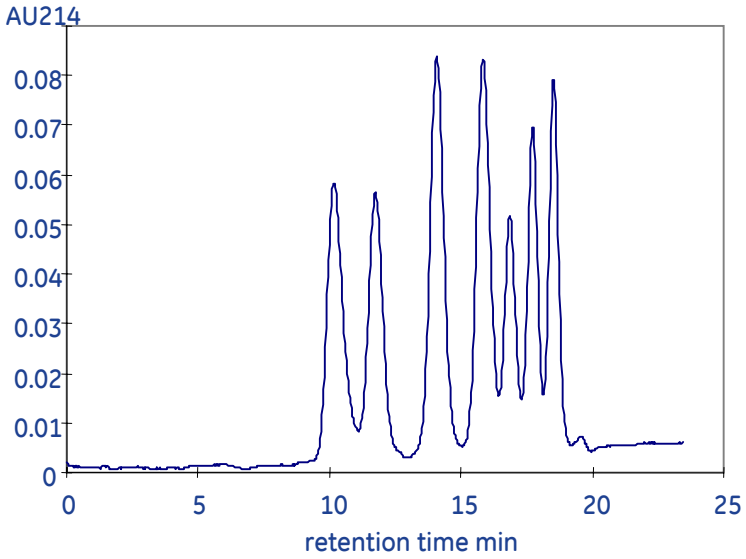
Resolution depends on column length
柱高影响分辨率

Increasing column length increases resolution
柱高加长，分辨率提高

2 x Superdex™ Peptide High Performance column 10x300 mm i.d x bed height



1 x Superdex Peptide High Performance column 10x300 mm i.d x bed height



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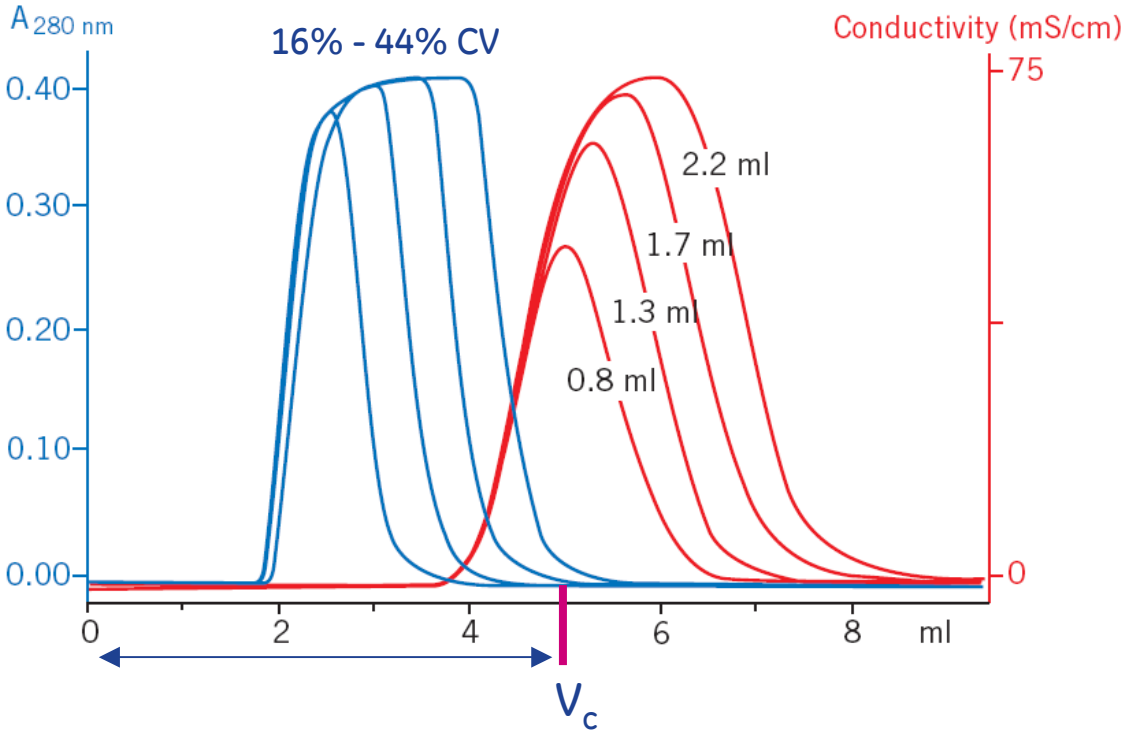
Main purification tasks 主要应用

- Group separations 组分离
  - –desalting, buffer exchange, removing reagents 脱盐/换缓冲液
- Fractionation of proteins and peptides 精细分离
  - –complex samples, monomer/dimer 聚集体去除
- Estimation size 测定分子量
- Characterisation: size homogeneity 蛋白鉴定：均一性

Group separations 组分离

Desalting proteins 蛋白质快速脱盐



Column: Hitrap desalting 5ml
Sample: HSA, 2 mg/ml,
Load: **0.8, 1.3**, 1.7, 2.2 ml **(16-26% Vt)**
Buffer: NaCl 0.5M

组分离: 柱高< 60cm, 进样体积较大(< 25% CV)

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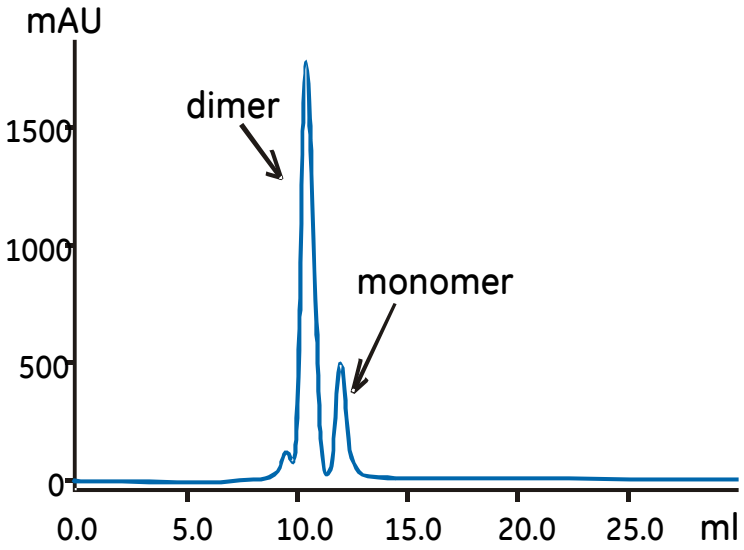
Fractionation 精细分离

Separating dimer and oligomers from monomer
去除蛋白聚集体



Column:	Superdex™ 75 10/300 GL Tricorn™
Sample:	recCys-prot., protein solution containing a mixture of dimers and monomers
Sample volume:	200 µl (**0.8% Vt**)
Elution buffer:	50mM TrisHCl, 1mM EDTA, 0.15M NaCl pH 8.4 filtered through 0.22 µm sterile filter
Flow rate:	0.5 ml/min (38cm/h)
System:	ÄKTAexplorer™ 100
Detection:	A_{280} nm

精细分离: 柱高30-100cm, 进样体积较小(一般 0.5-5 % CV)

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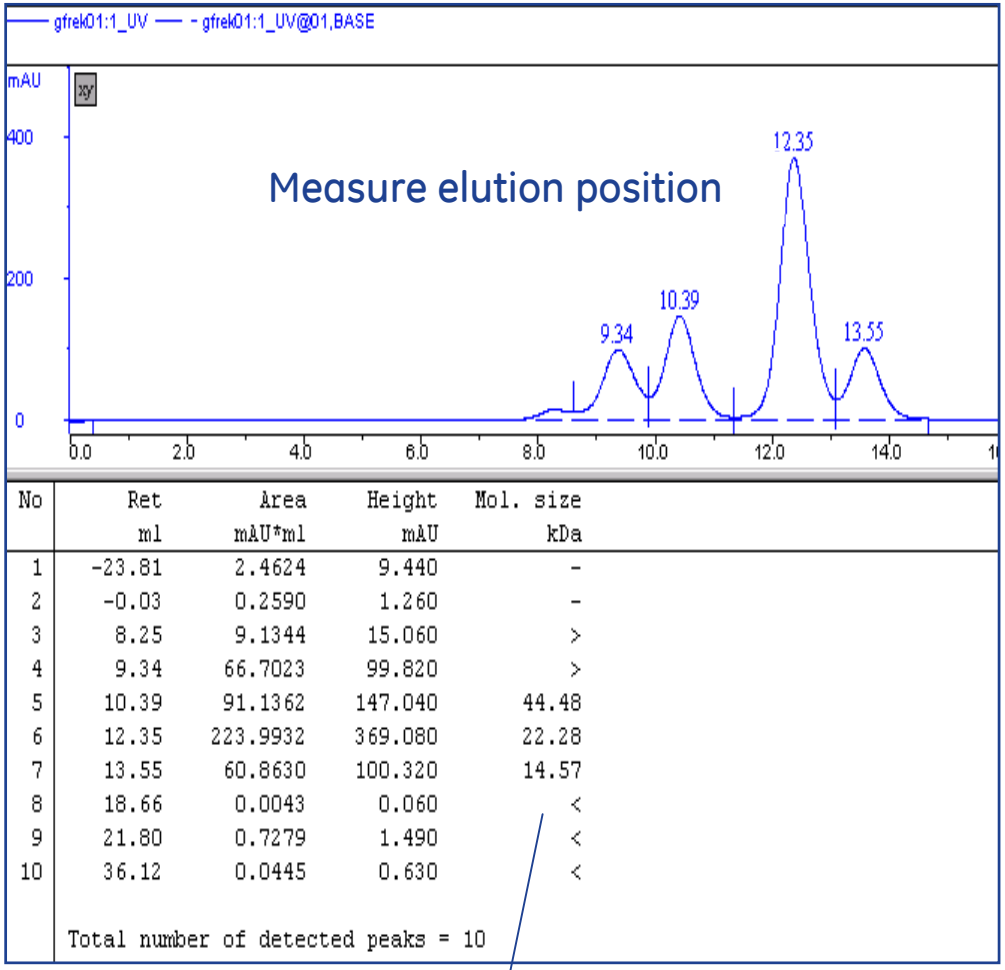
Estimating molecular size 测定分子量

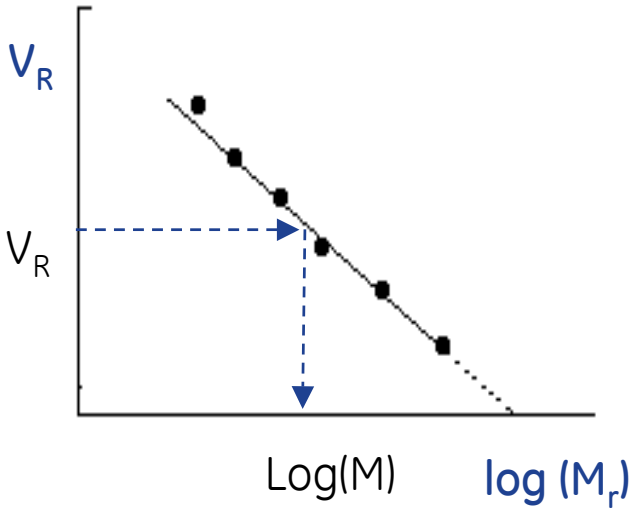


No	Ret ml	Area mAU*ml	Height mAU	Mol. size kDa
1	-23.81	2.4624	9.440	-
2	-0.03	0.2590	1.260	-
3	8.25	9.1344	15.060	>
4	9.34	66.7023	99.820	>
5	10.39	91.1362	147.040	44.48
6	12.35	223.9932	369.080	22.28
7	13.55	60.8630	100.320	14.57
8	18.66	0.0043	0.060	<
9	21.80	0.7279	1.490	<
10	36.12	0.0445	0.630	<

Total number of detected peaks = 10

计算分子量大小

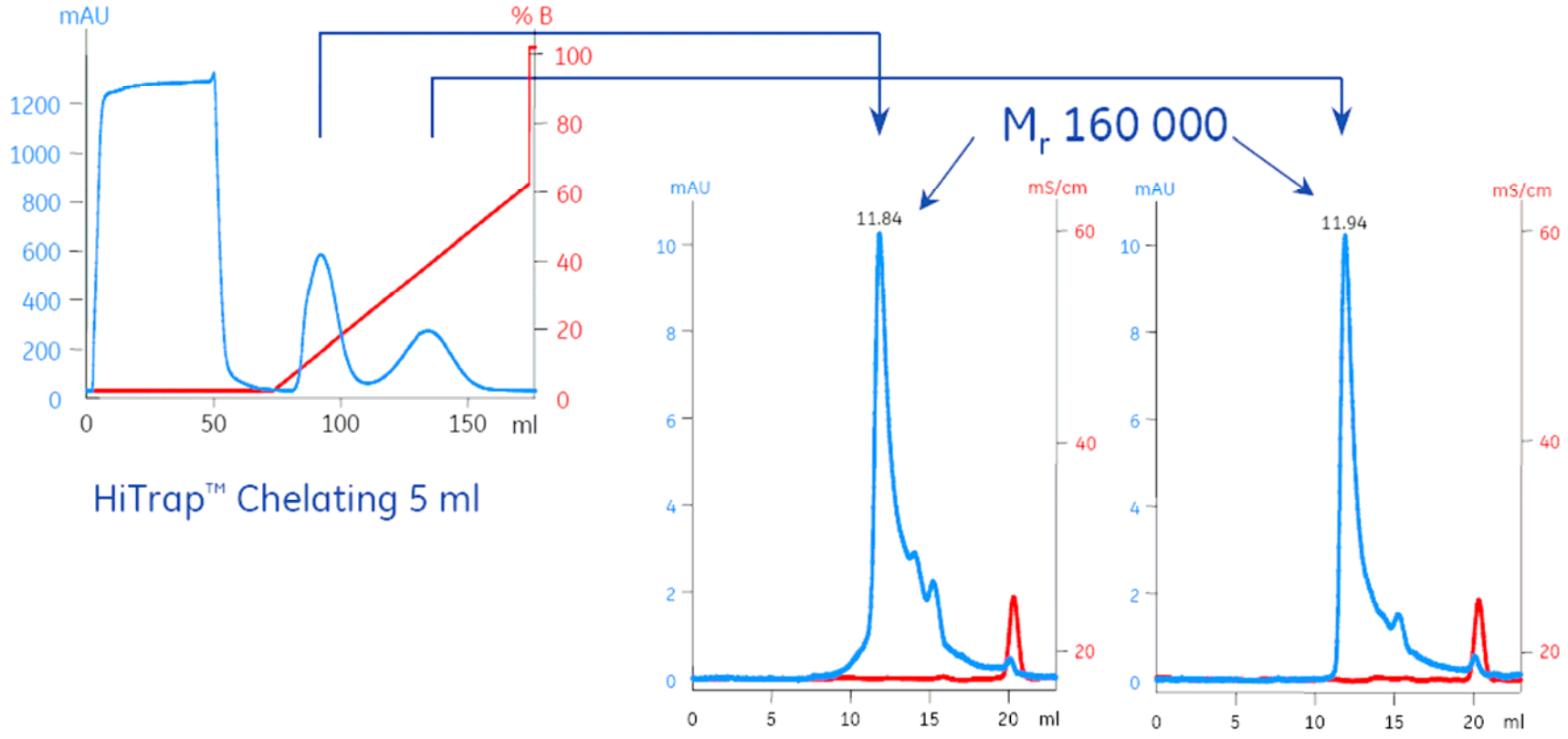
Calibration Curve
校准曲线



$V_R \sim Log\ (M_r)$

imagination at work

Characterisation: size homogeneity
蛋白鉴定：均一程度



HiTrap™ Chelating 5 ml

Superdex™ 200 HR 10/30

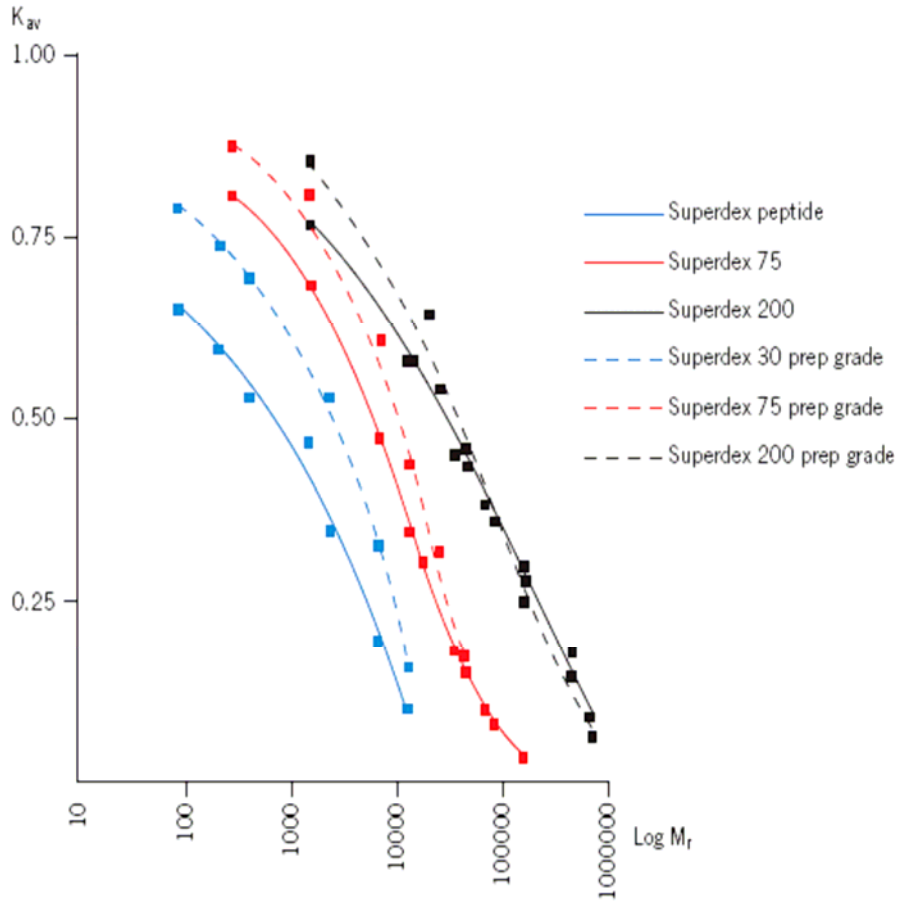
imagination at work

Main products 主要产品

- Superdex : 首选的分子筛凝胶
  - Highest selectivity选择性高, high speed快速, scale-up可放大
- Sephacryl HR
  - complex samples, monomer/dimes聚集体去除, 小规模
- Superose
- Sepharose
  - Virus, pDNA, polysaccharides,病毒/DNA/多糖 etc
- Sephadex
  - Fast desalting, buffer exchange, removing reagents
    快速脱盐，置换缓冲液，去除试剂

Superdex：首选的分子筛填料

- 葡聚糖和琼脂糖交联而成
- 高选择性，高分辨率
- 速度快，吸附最低，收率高
- 化学性质稳定
- 流速快，耐压性能好



常用于对分辨率要求比较高的精细纯化!

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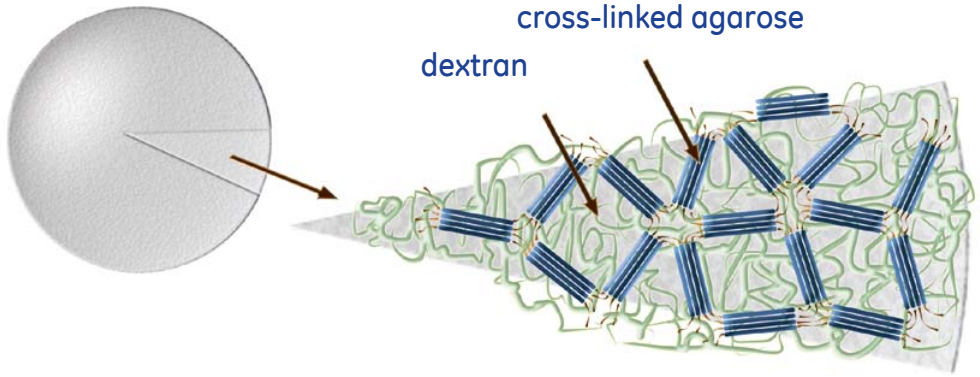
Gel structure 填料结构

A hypothetical structure for Superdex



cross-section from Superdex™ particle

Cross-linked agarose
交联琼脂糖

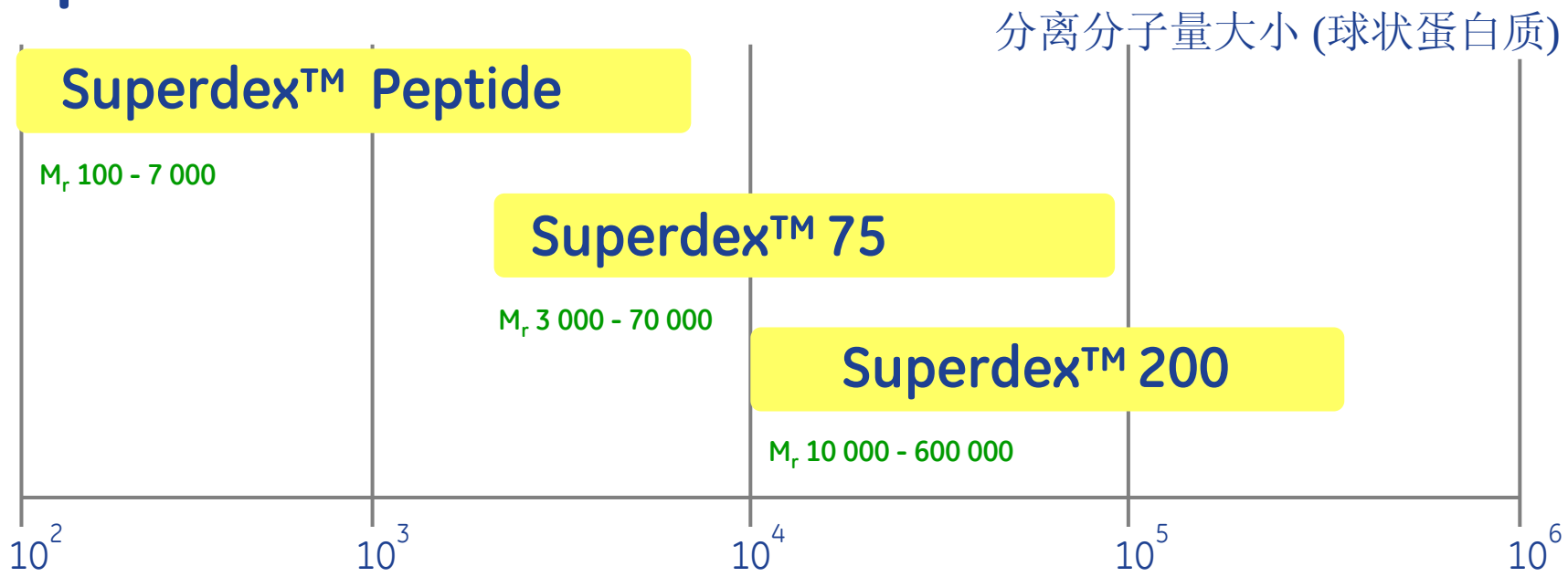
strong support
支撑

Dextran chain
葡聚糖链

controlled pore size for separation
控制孔径

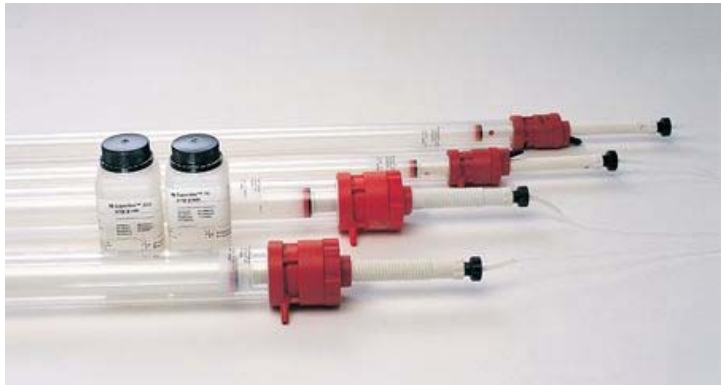
imagination at work

Superdex



Tricorn Superdex 10/30GL_24ml

13um



Hiload Superdex 16/26 120ml /320ml

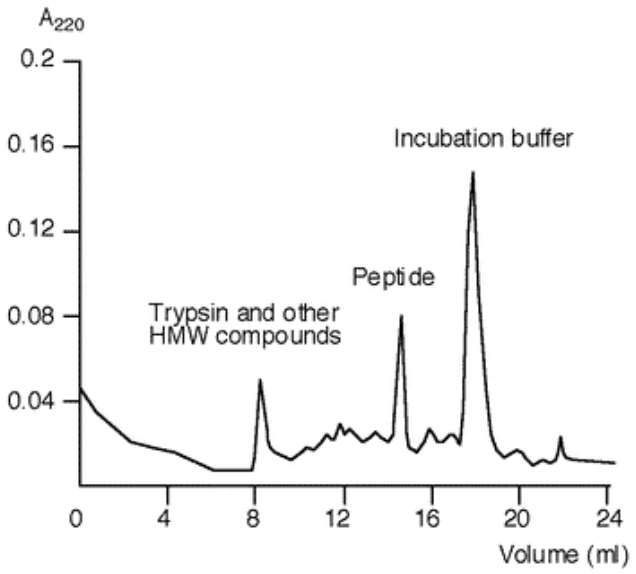
34um

imagination at work

Peptide Purification 多肽纯化

Preparation of digestion fragments by SEC

Sample: Trypsin-treated recombinant phage
Sample volume: 10 µl
Column: Superdex Peptide HR 10/30
Eluent: Sodium phosphate buffer (50 mM, pH 7.0)
Flow rate: 0.2 ml/min
Detection: 220 nm

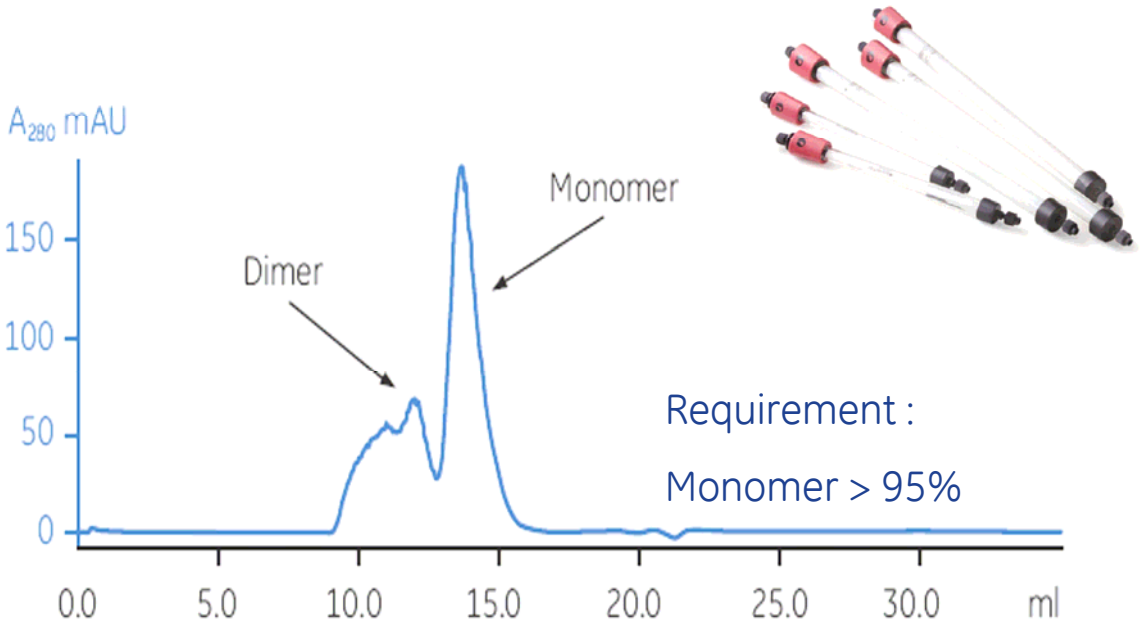


Superdex Peptide

- 分离范围100-7000 Da
- 高化学稳定性, pH1-14, 兼容有机溶剂 (乙腈+TFA)
- 和RPC衔接, 用于peptide的高效分离纯化

imagination at work

Aggregates Removal 聚集体去除



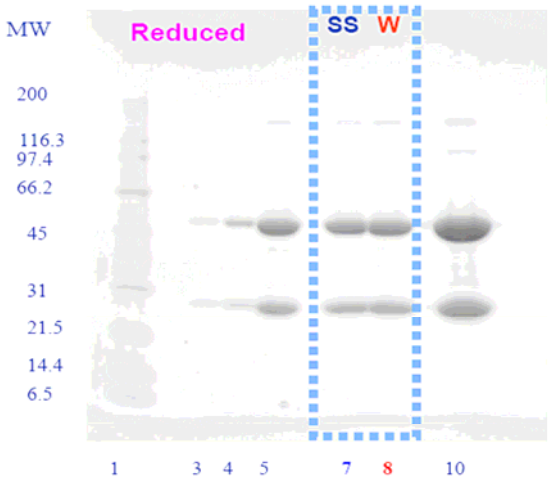
Requirement :

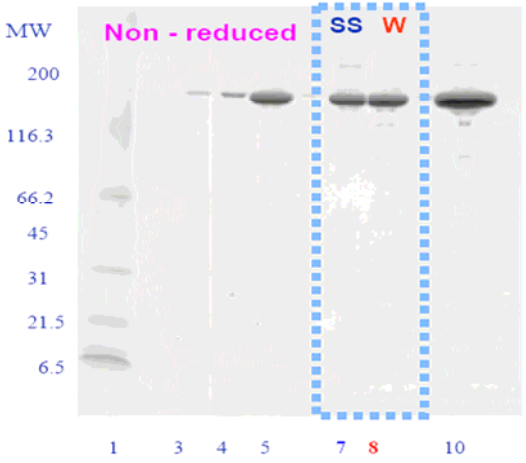
Monomer > 95%

Fig 7. Separation of the monomer and dimer of a monoclonal antibody on Superdex 200 10/300 GL.

Superdex 200 10/300GL

SDS-PAGE





imagination at work

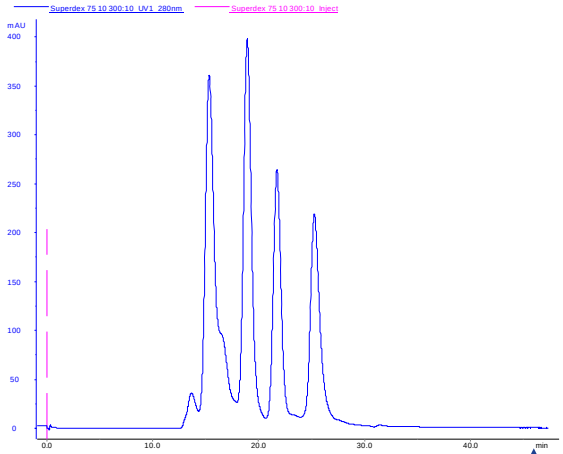
比较 Superdex™ 75 5/150 GL & Superdex 75 10/300 GL

Superdex 75 10/300 GL

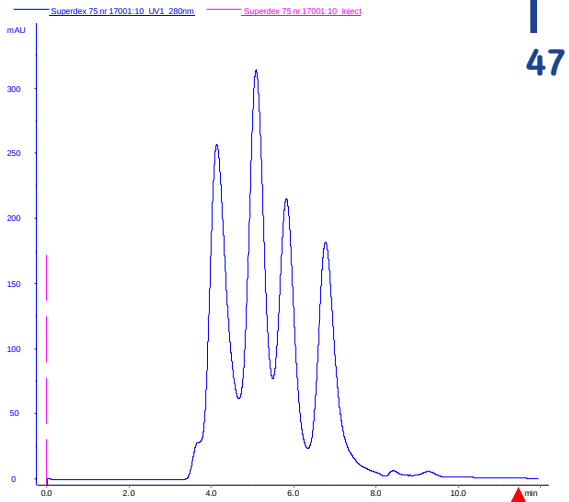
Column volume	24 ml
Flow rate	0.6 ml/min
Sample volume	**100 µl**
Analysis time	**47 min**

Superdex 75 5/150 GL

Column volume	3 ml
Flow rate	0.3 ml/min
Sample volume	**12 µl**
Analysis time	**12 min**



47 min



12 min!

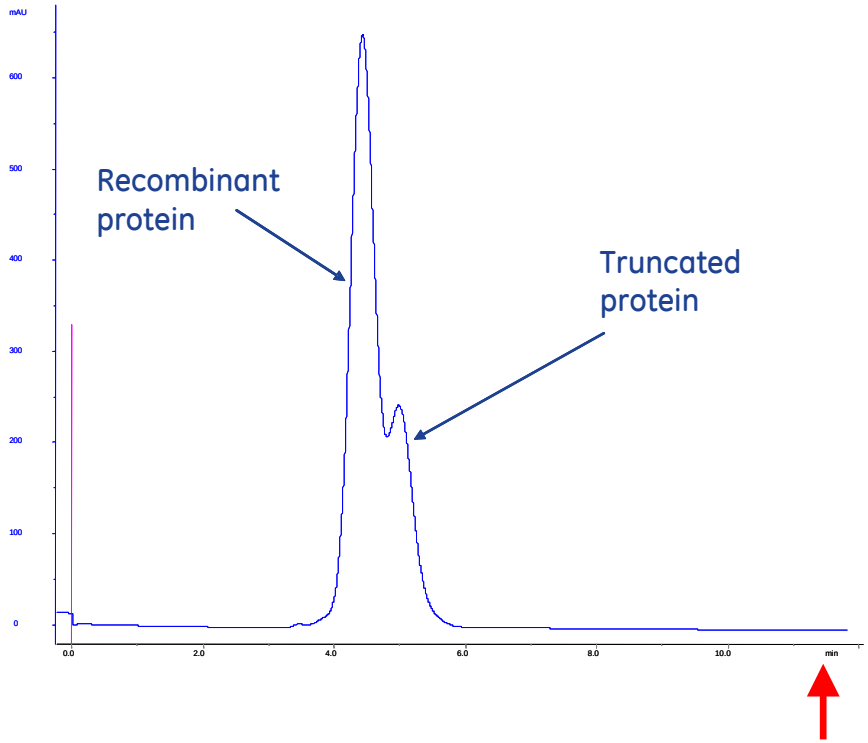
快速得到好的分辨率!

imagination at work

Rapid purity check of r-protein
重组蛋白纯度快速检测

Recombinant protein ~17 kDa.
Truncated form ~10 kDa.

Column:	**Superdex™ 75 5/150 GL**
Buffer:	PBS pH 7.4
Flow rate:	0.3 ml/min
Sample volume:	4 µl
Analysis time:	12 min/run
System:	Ettan™ LC



- 样品纯度检测非常快
- 可以粗略估测纯度
- 精确的定量分析请选择 10/300 GL

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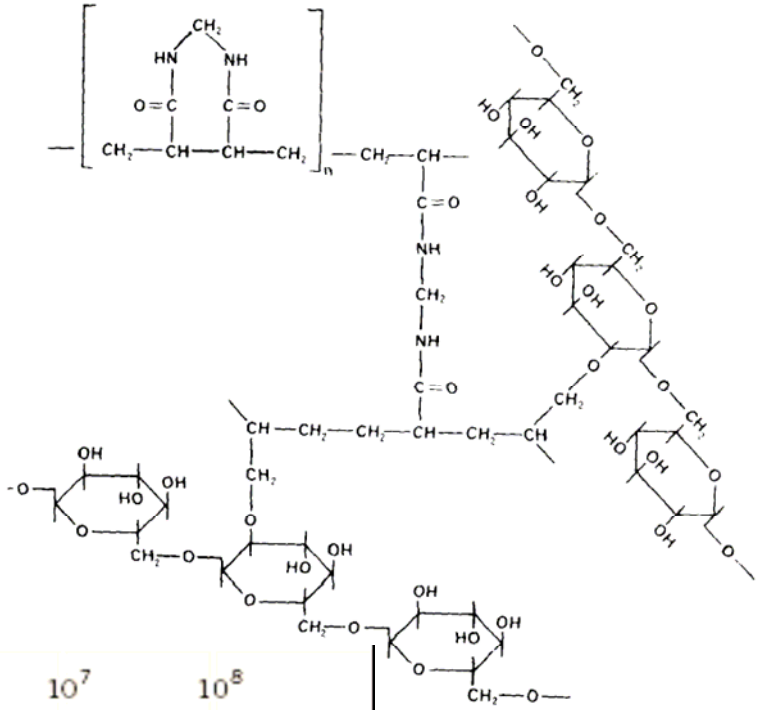
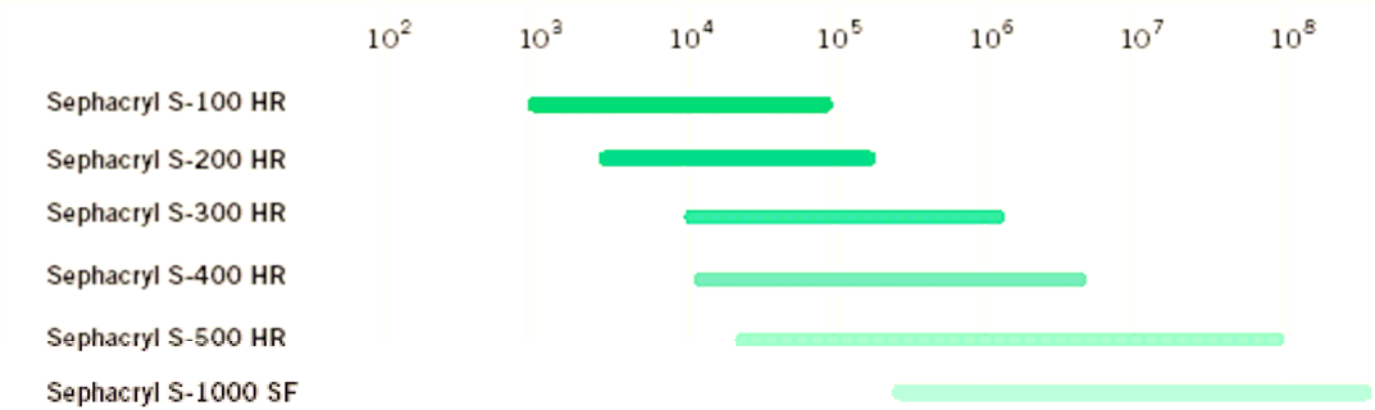
Sephacryl HR

-由葡聚糖交联N,N－亚甲基二乙酰胺形成

- 理化性质稳定

- 系列全, 成本低





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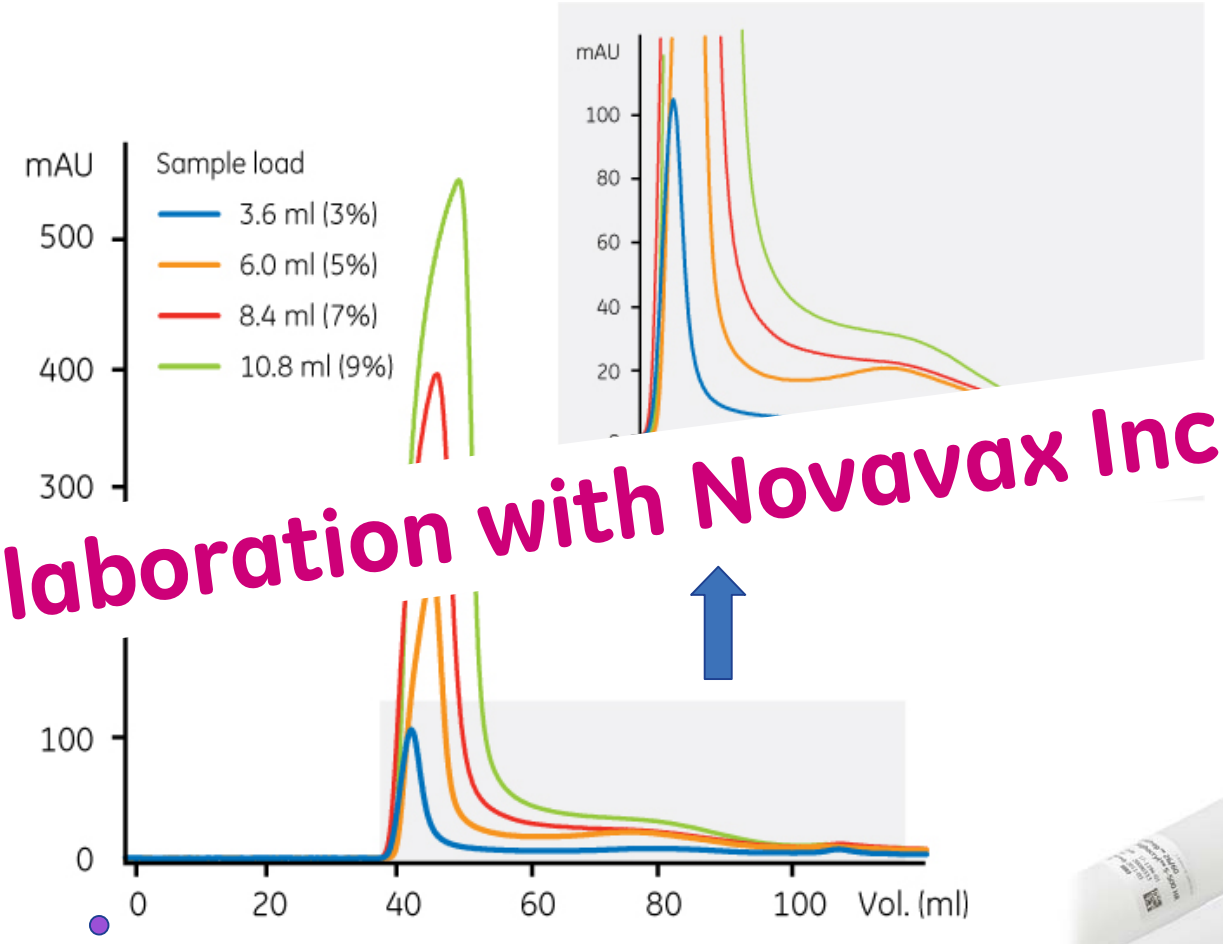
类病毒颗粒的纯化 (VLP)

HiPrep™ 16/60 Sephacryl™ S-500 HR



Collaboration with Novavax Inc., US

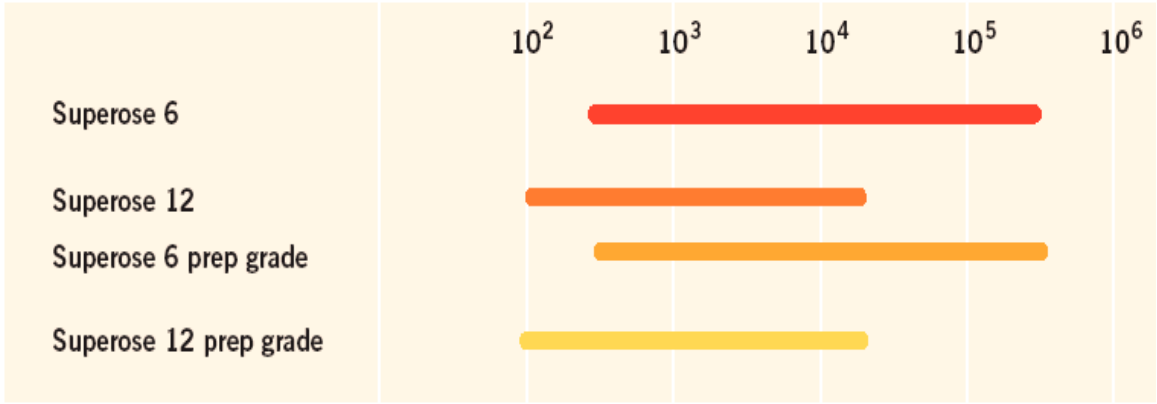
imagination at work

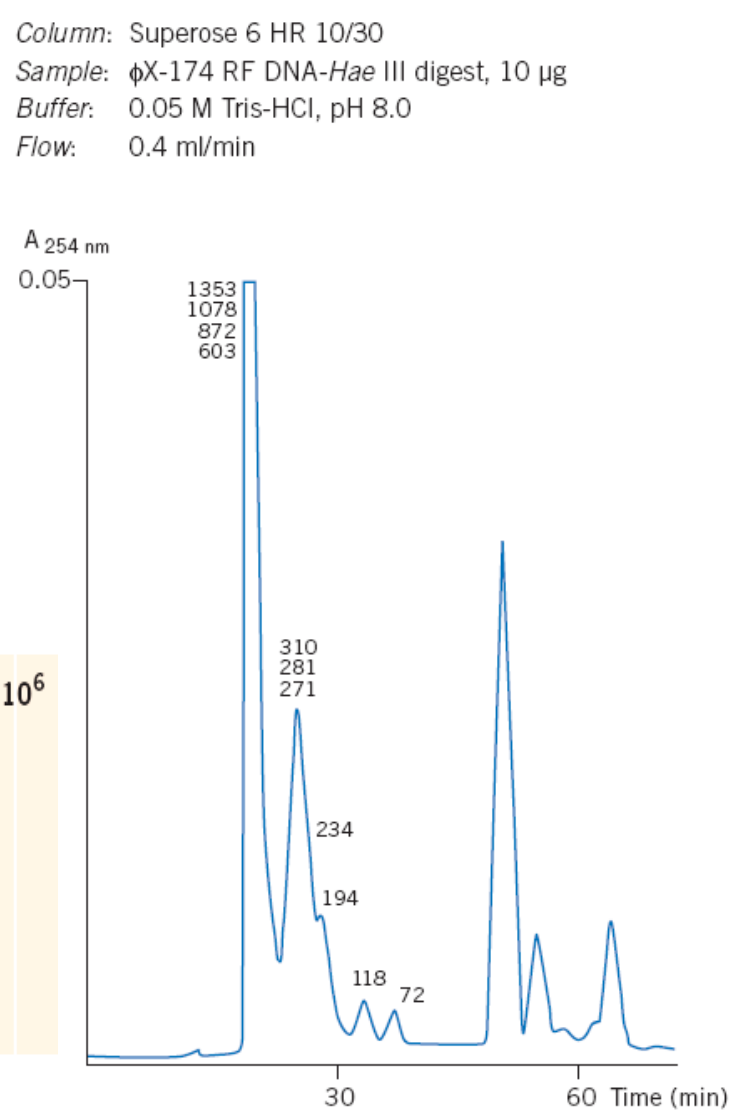
Superose

-高度交联的琼脂糖

-分离范围广

-理化性质稳定





imagination at work

Sepharose

- 交联的琼脂糖
- 理化性质稳定
- Fast Flow 填料流速快：250-300cm/h

  Sepharose 4FF: 60kD~20MD

  Sepharose 6FF: 10kD~4MD
- 常用于疫苗领域

Influ.& Rabies Vaccine Production
流感 & 狂犬等病毒类疫苗的生产

Cell culture supernatant

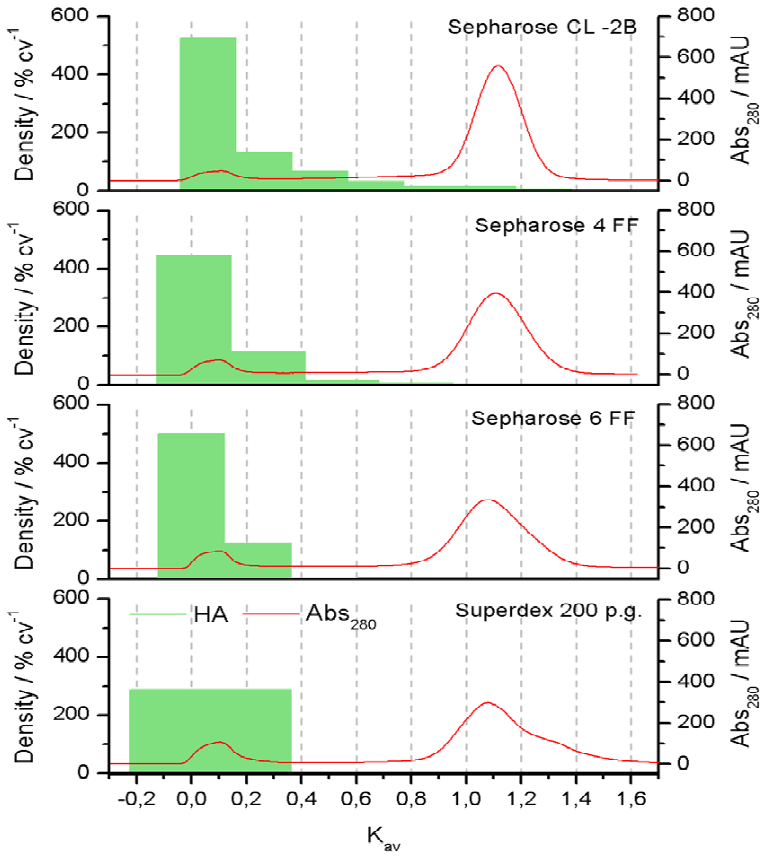
750k ultrafiltration

SEC: Sepharose 4FF

AEX: Q Sepharose FF

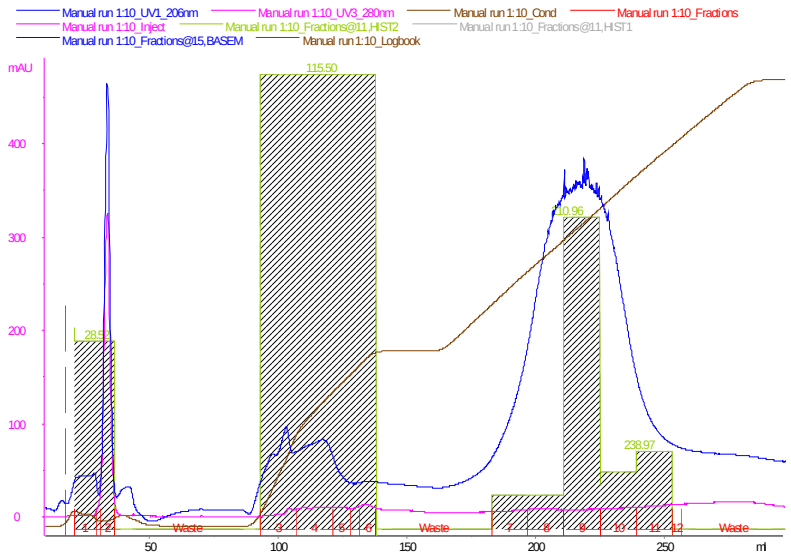
ultra-/diafiltration

purified human influenza virus

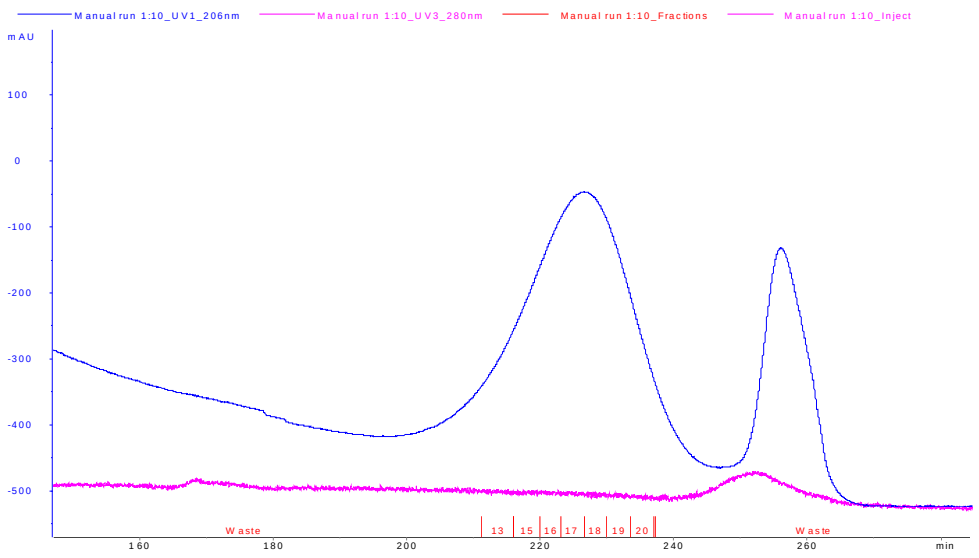


imagination at work

多糖疫苗：两步层析代替酚抽提

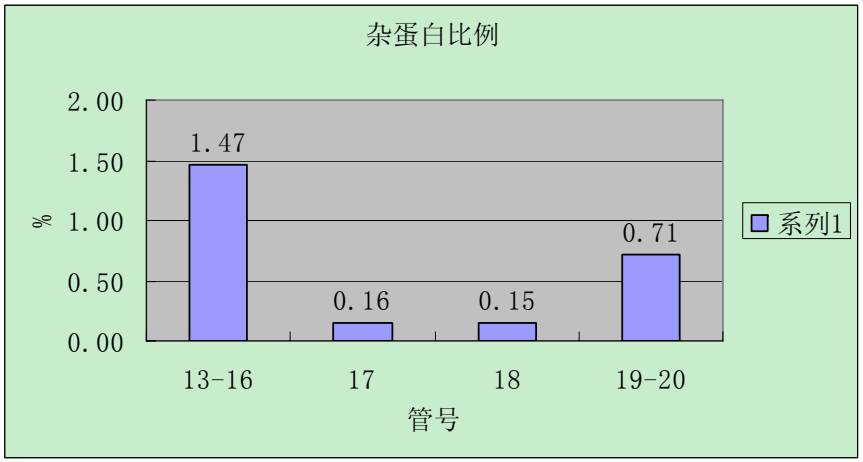


阴离子交换 Capto adhere



Sepharose 4FF

终产品蛋白残留 < 1%



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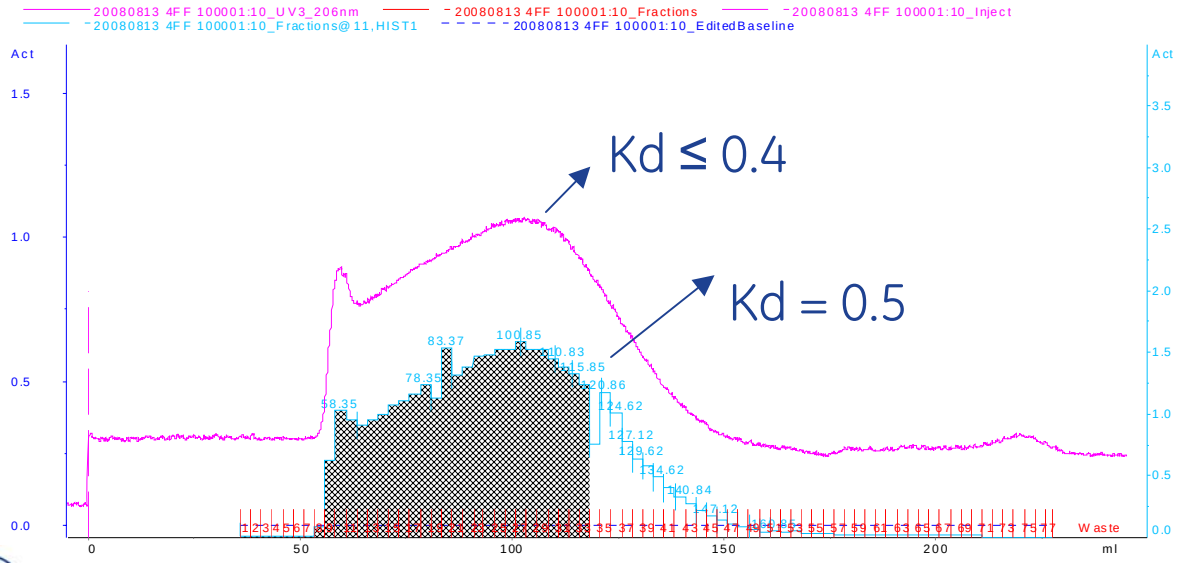
多糖疫苗的检定

药典CP2005：

用琼脂糖-4B或CL-4B凝胶过滤法测定，K_D 值应≤0.40（即retention2处的Kd2值≤0.40）。K_D值表示相对分子量，多糖抗原分子量要大于10kd才具有免疫原性。



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超螺旋质粒pDNA纯化

Clarified alkaline lysate
裂解液

HiTrap™ 26/10 **Sepharose ™ 6 FF**
26 mm x 100 mm
Vt: 53 ml

RNA-removal

HiTrap PlasmidSelect Xtra
16 mm x 25 mm
Vt: 5 ml

Supercoiled pDNA capture

HiTrap SOURCE 30Q
16 mm x 25 mm
Vt: 5 ml

Supercoiled pDNA polishing

Purified supercoiled pDNA
纯化的pDNA

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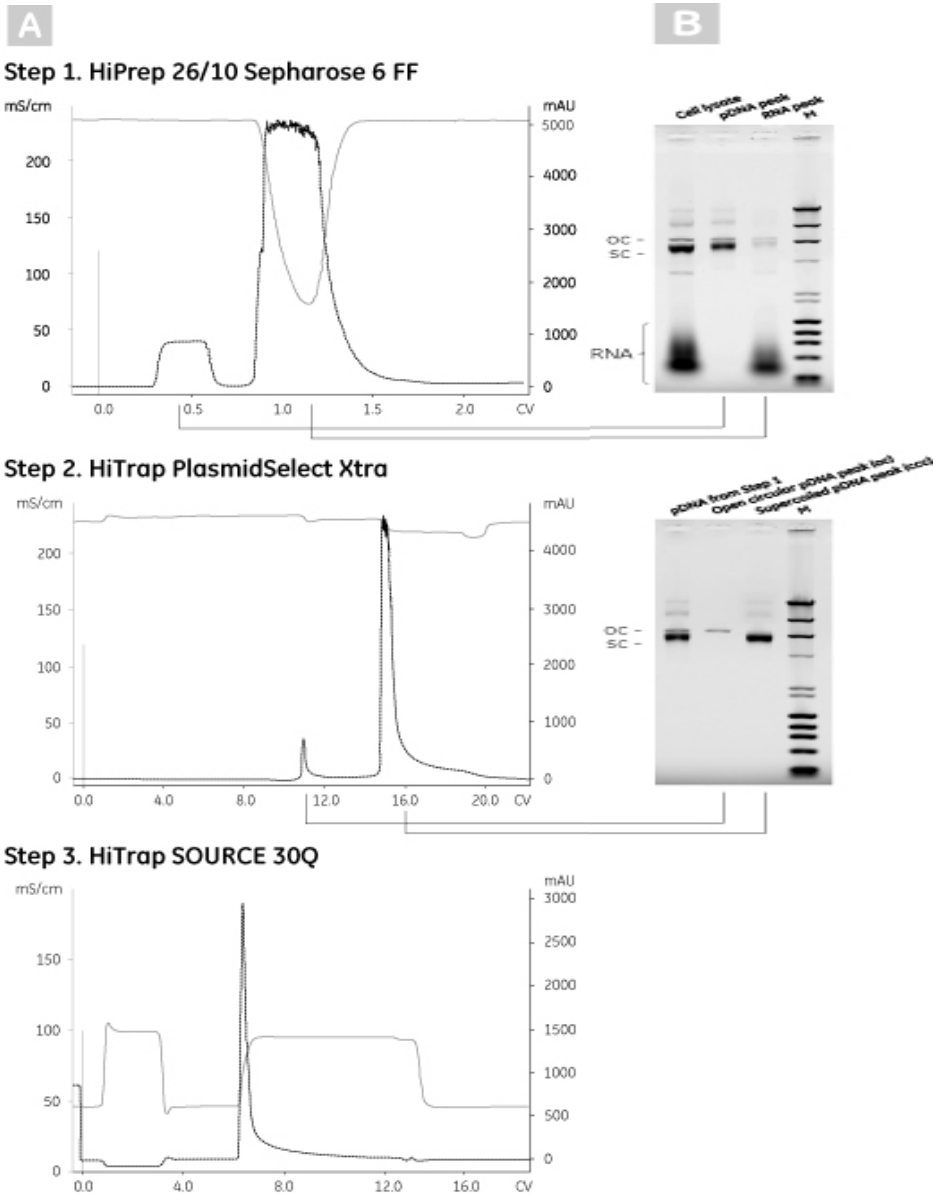
PlasmidSelect Xtra Starter Kit



Sephadex

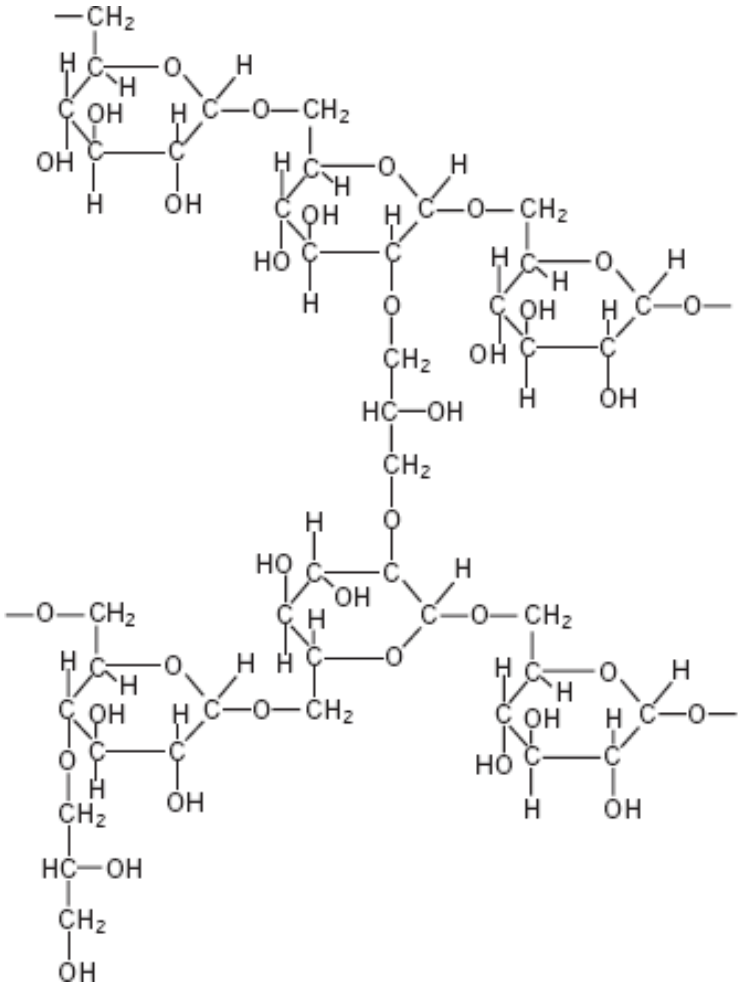
-由葡聚糖交联异表氯醇形成的多孔结构

- Sephadex G10: 100-700 Da

多肽纯化, oligo脱盐, 抗生素聚集体质控分析

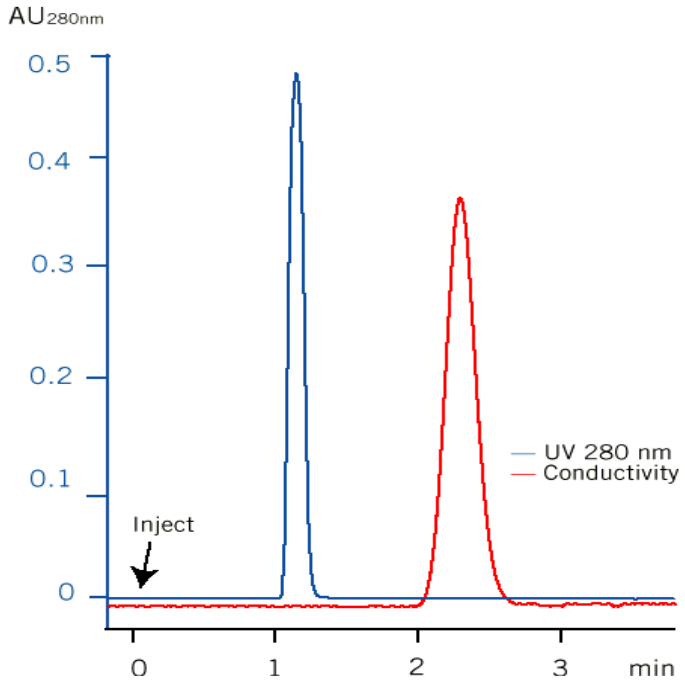
-Sephadex G25: 1k– 5kDa

MW>5kD 蛋白快速脱盐



Buffer exchange using 使用脱盐柱快速脱盐 HiPrep 26/10 Desalting Column

Sample: BSA dissolved in 50 mM piperazine, 0.5 M sodium chloride, pH 6.2
Column: HiPrep™ 26/10 Desalting_53ml
Buffer: 20 mM sodium phosphate, 0.15 M sodium chloride, pH 7.0
System: ÄKTAprime™, 20 ml/min



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Content 内容

Principles of gel filtration 凝胶过滤原理

Optimize : How to get the expected results 优化

Products and applications 产品和应用

Practical considerations 实际需考虑的因素

Summary 总结

4 key questions 4个问题

1. What is the aim of the experiment? 实验目的？
- High resolution fractionation 精细分离
  or 还是
- Group separation (desalting, buffer exchange)? 组分离，缓冲液置换？

High resolution fractionatio精细分离: Superdex™, Superose™, Sephacryl™

Group separation组分离: Sephadex™

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4 key questions 4个问题

2. What is the molecular weight of the target protein/contaminants? 目标蛋白和杂质的分子量

Choose a gel filtration medium with the fractionation range that covers the molecular weight values of interest in your sample and that gives highest difference in size of target protein/contaminants/salt

选择分级范围能包含目标蛋白，且使杂质和目标蛋白的分离度最大的填料

4 key questions 4个问题

3. How much sample are they loading? 进样量是多少?

Desalting 脱盐 25% CV

PD-10 (gravity) loading capacity 2.5 ml

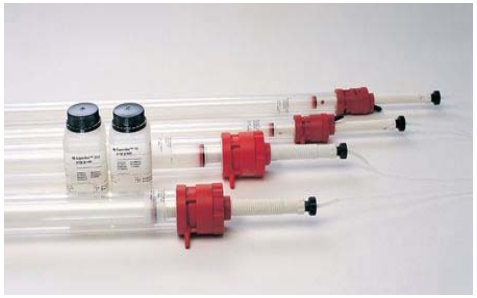
HiTrap™ 5 ml loading capacity 1.5 ml

HiPrep™ 53 ml loading capacity 15 ml

Fractionation – polishing 精细纯化

µl – analytical column 分析 1%CV

- 10/300 loading capacity 250 µl
- 5/150 loading capacity 50 µl



ml – preparative column 制备 5%CV

- 16/60 loading capacity 5 ml
- 26/60 loading capacity 13 ml

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更大规模，可以自行填装柱子

4 key questions 4个问题

4. Will you be scaling up? 是否打算放大

Superdex™ - $$$ (1st choice 首选)

Sephadex™ - $ (desalting 脱盐)

Sephacryl™ - $$ (large column packing is difficult 放大较困难)

Appropriate industrial chromatography system and column available?

是否有合适的工业层析系统和层析柱？

如何得到满意的结果？

- 使用选择性最高的填料!
- 使用预装柱!
- 尽量使用粒径小而均一的填料
- 合理的进样量/柱高
  - Group separation 组分离: < 25% CV, 短
  - Fractionation 精细分离 : 0.5-5% CV, 30-100cm
- 注意样品黏度
- 降低流速以改善传质速度 (通常5-20cm/h)
- 流动相中推荐使用150mM NaCl

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Summary 总结

Pros 优点

- Simple 简单
- Separates by size 根据大小分离
- Fast method for buffer exchange
  快速缓冲液置换
- Very gentle, high yields
  温和, 高收率
- Works in any buffer solution
  buffer没有特殊要求
- Removes dimers and aggregates
  去除聚集体

Cons 缺点

- Limited sample volume
  处理体积有限
- Dilute the sample
  会产生一定的稀释
- Low flow rate
  流速慢, 分离时间长
- Scale-up
  大规模放大有一定的困难

Q & A

Please visit:

www.gehealthcare.com/protein-purification

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