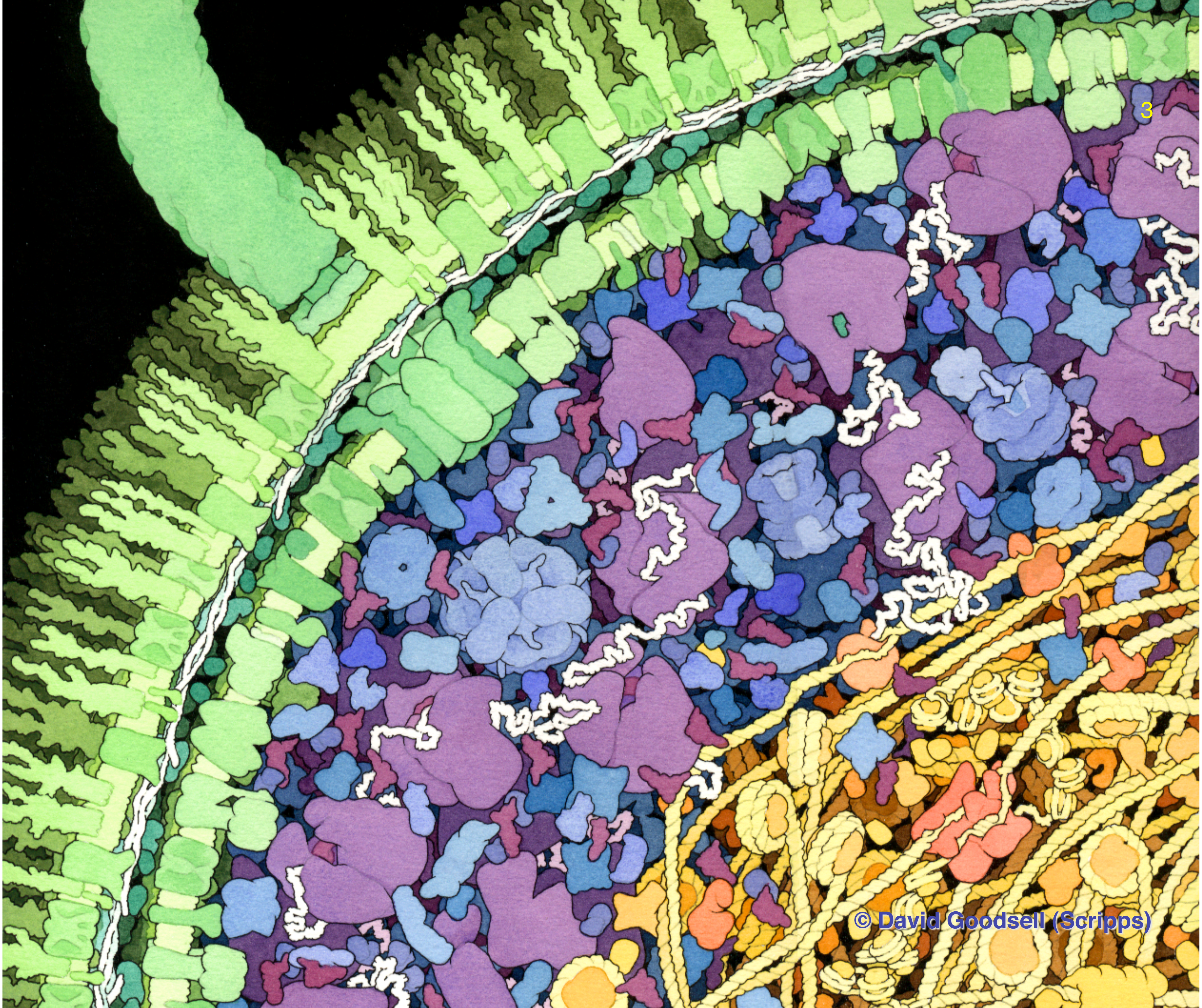
Module 2 overview

lecture	*lab*
1. Introduction to the module	1. Start-up protein eng.
2. Rational protein design	2. Site-directed mutagenesis
3. Fluorescence and sensors	3. DNA amplification
4. Protein expression	4. Prepare expression system
SPRING BREAK	
5. Review & gene analysis	5. Gene analysis & induction
6. Purification and protein analysis	6. Characterize expression
7. Binding & affinity measurements	7. Assay protein behavior
8. High throughput engineering	8. Data analysis

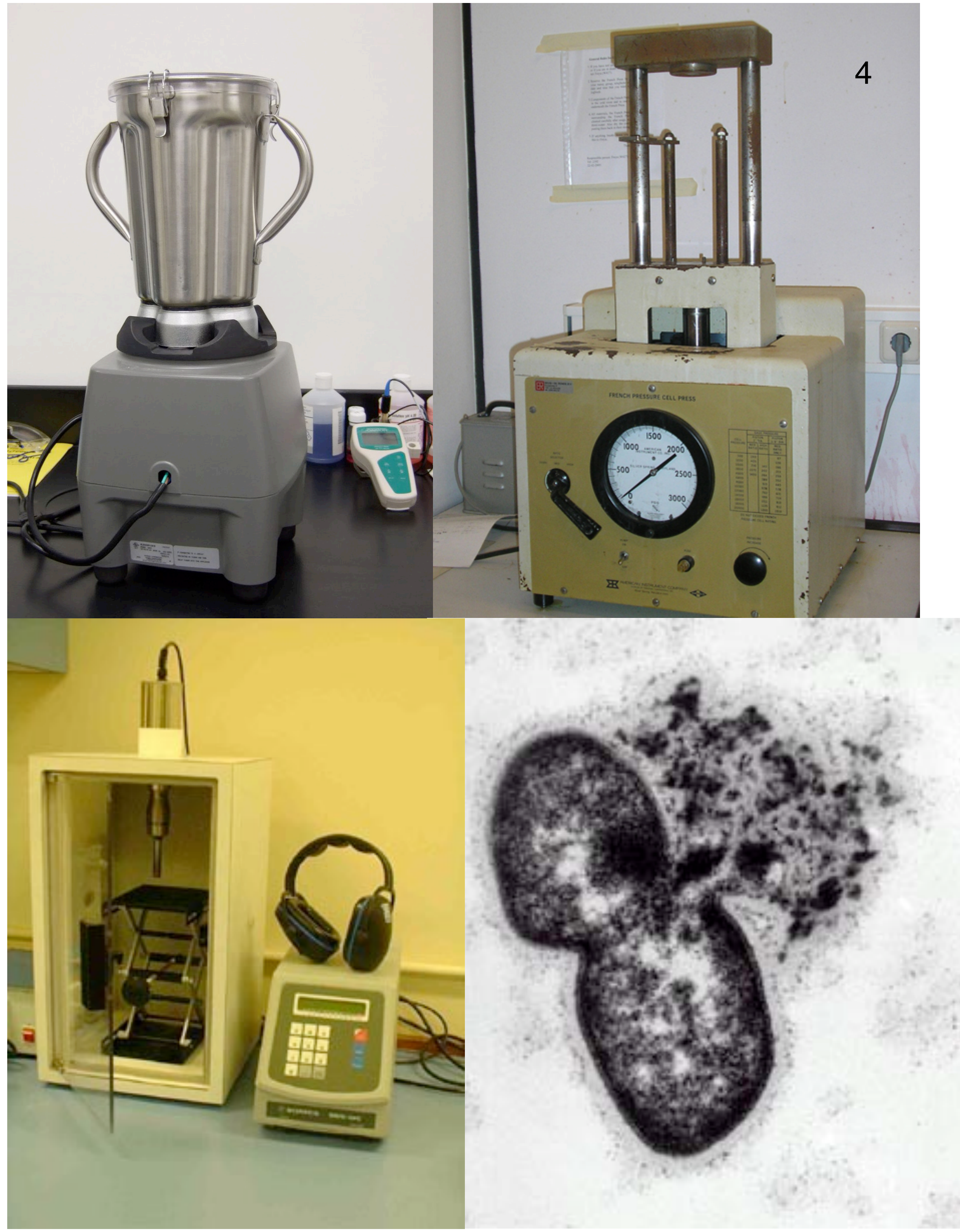
Lecture 6: Protein purification

I. Standard purification methods
   A. Harvesting and lysis
   B. Protein separation techniques

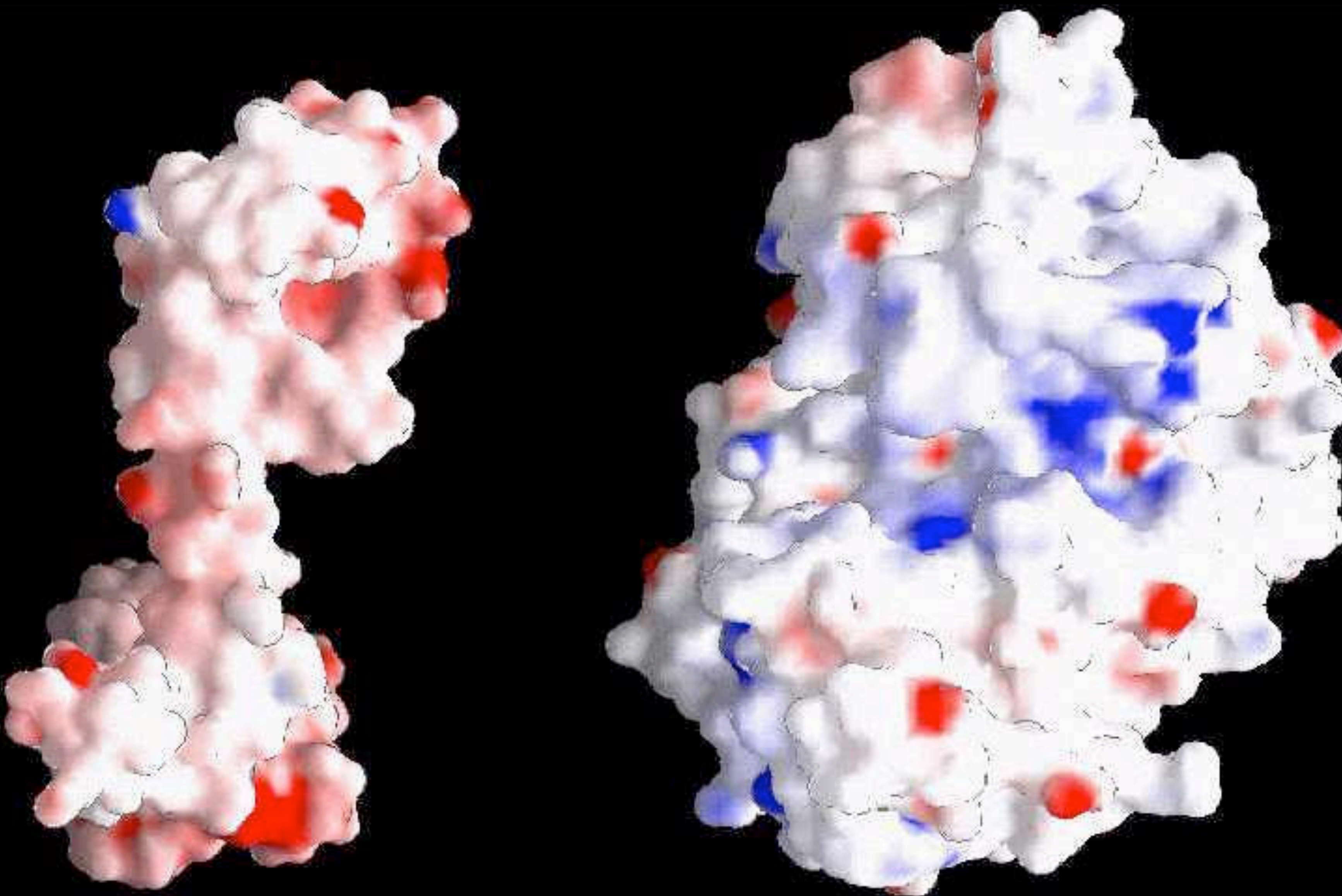
II. Assessing purified proteins
   A. Electrophoresis
   B. Mass spectrometry
   C. Protein sequencing and AA analysis


© David Goodsell (Scripps)

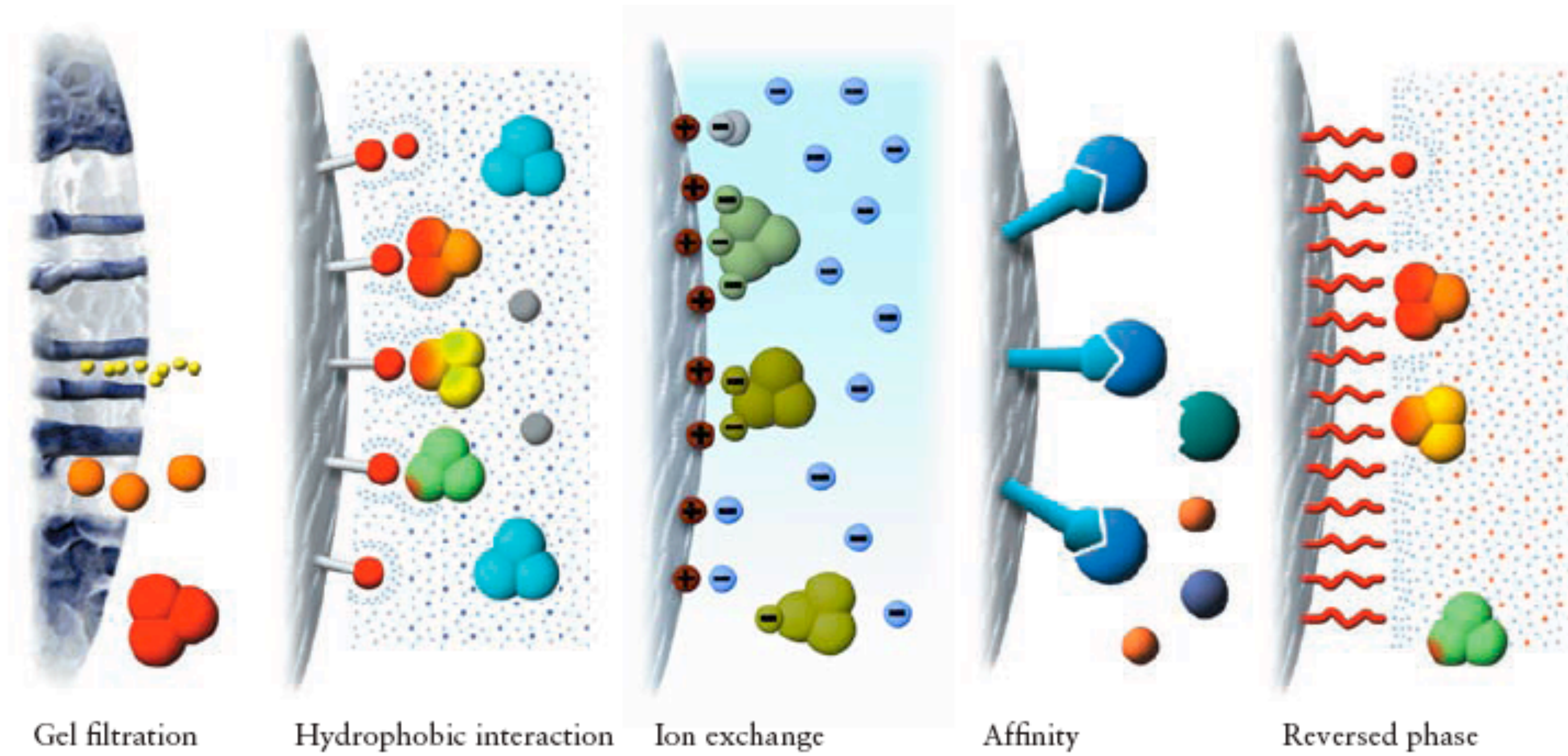
Once we've collected the cells, how do we get the proteins out?



clockwise from top left:
lh6.ggpht.com
www.biomembranes.nl
bioinfo.bact.wisc.edu
matcmadison.edu



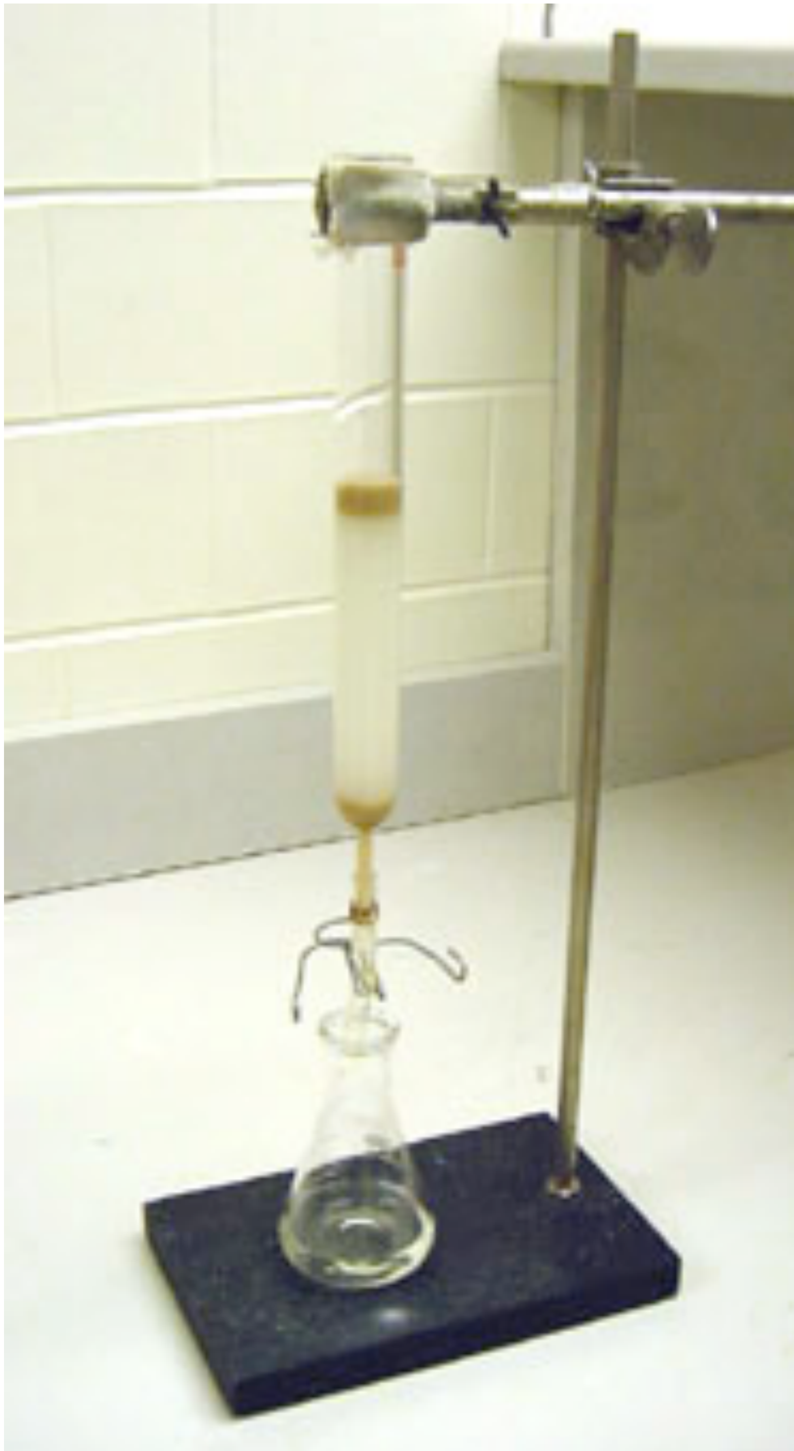
Separation techniques



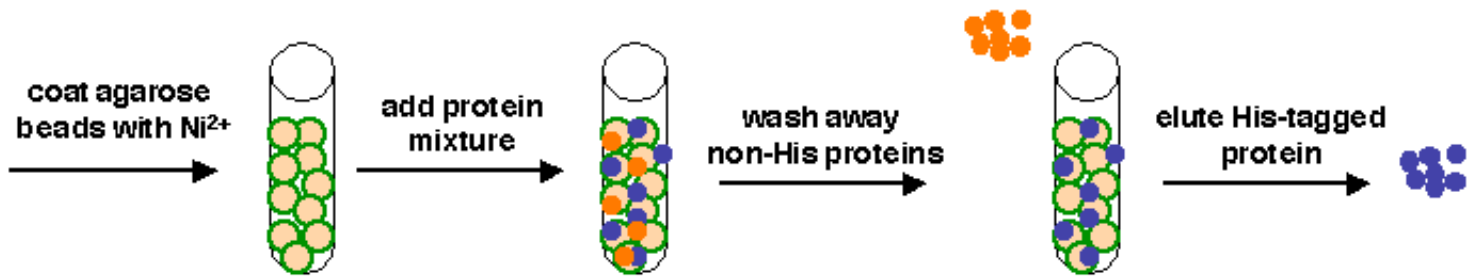
most common, in addition to affinity

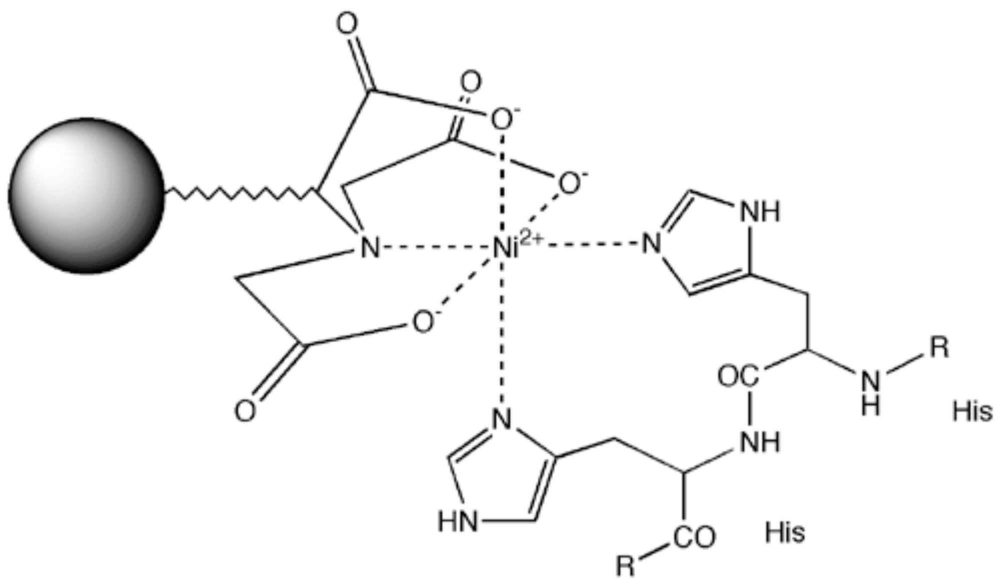
***e.g.* Ni-NTA**

GE Healthcare (2007) *Recombinant Protein Purification Handbook*



Nickel affinity purification with Ni-NTA agarose





Many other tags can be used for protein purification:

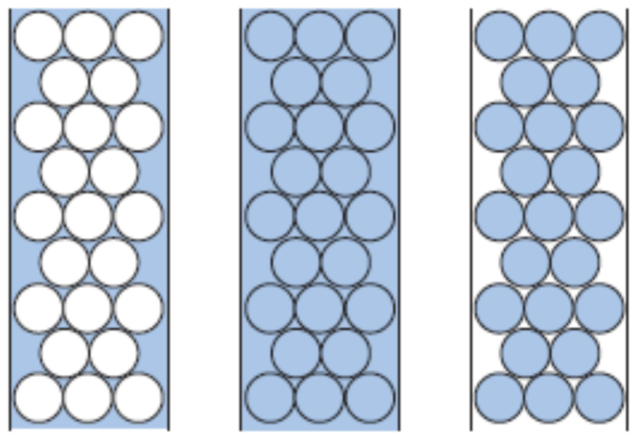
tag	*residues*	*matrix*	*elution condition*
poly-His	~6	Ni-NTA	imidazole, low pH
FLAG	8	anti-FLAG antibody	low pH, 2-5 mM EDTA
c-myc	11	anti-myc antibody	low pH
strep-tag	8	modified streptavidin	2.5 mM desthiobiotin
CBP	26	calmodulin	EGTA, EDTA
GST	211	glutathione	reduced glutathione
MBP	396	amylose	10 mM maltose

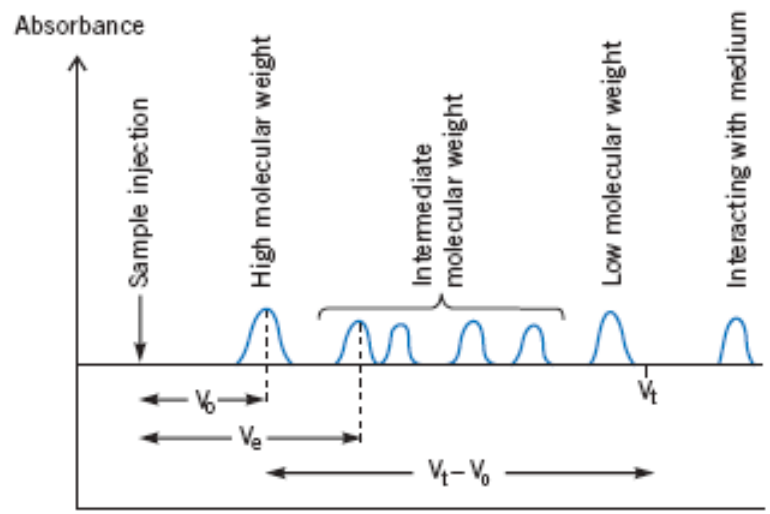
Tags may be chosen because they

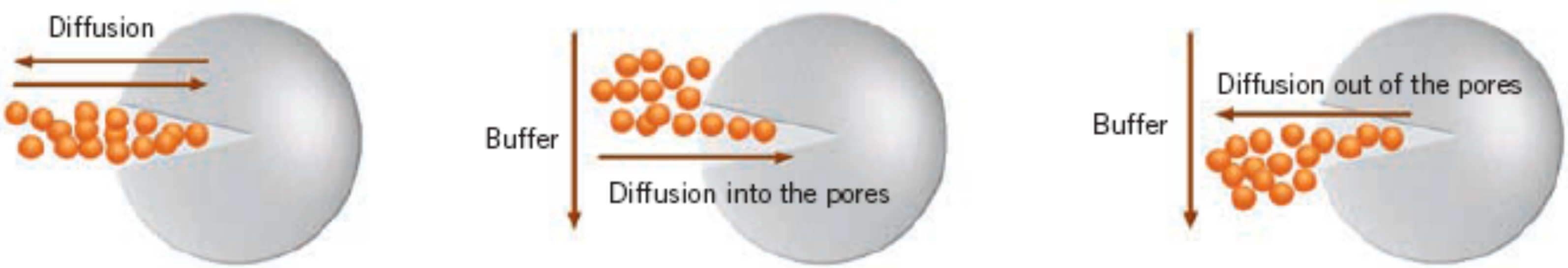
- interfere minimally with protein structure/function
- improve recombinant protein expression or solubility
- offer most convenient purification methods

All tags may be cleaved from expressed proteins using specific proteases, if desired.

Gel filtration (size exclusion chromatography) principle

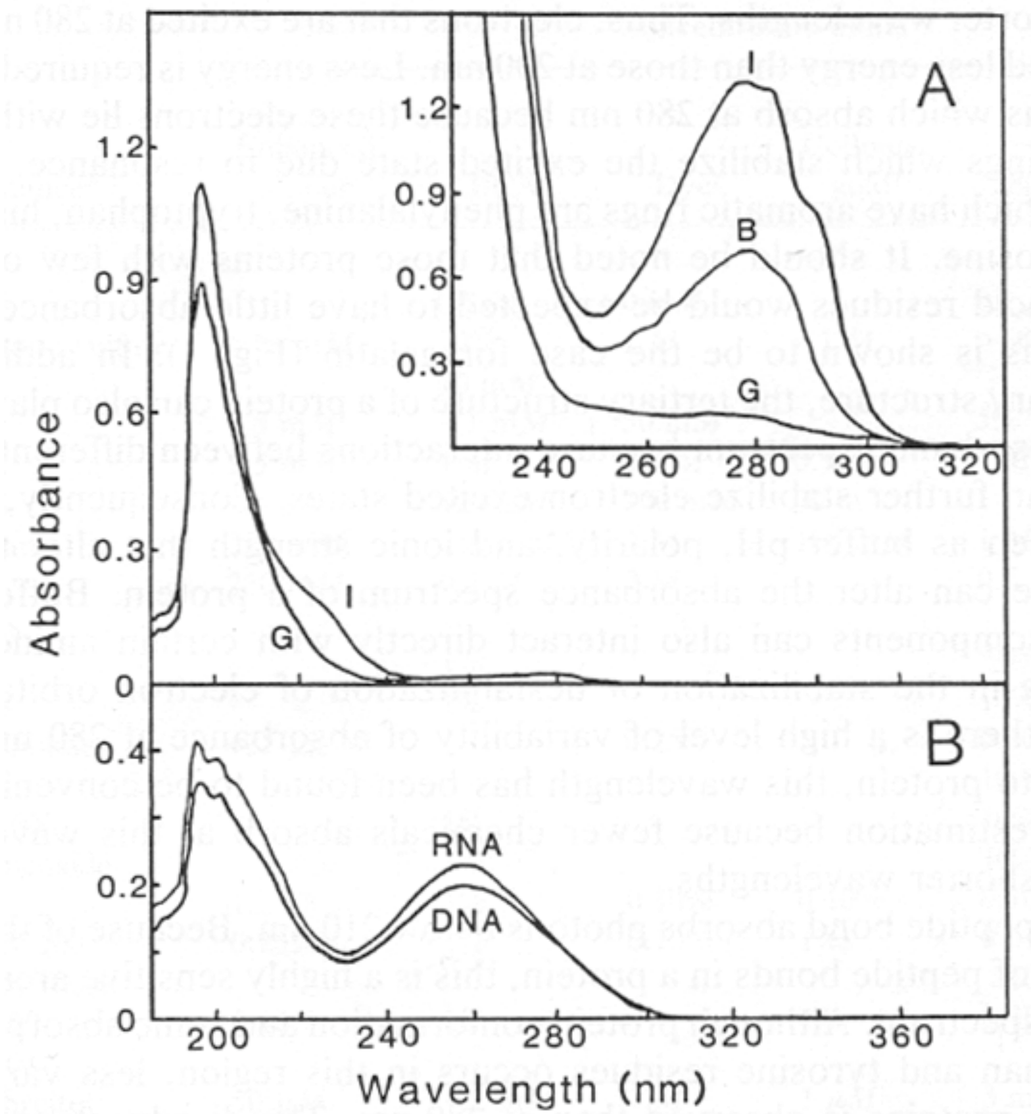






GE Healthcare (2007) *Gel Filtration Principles & Methods*

Quantification of purified proteins



use Beer-Lambert law:

$$A_{280} = \varepsilon_{280} c l$$

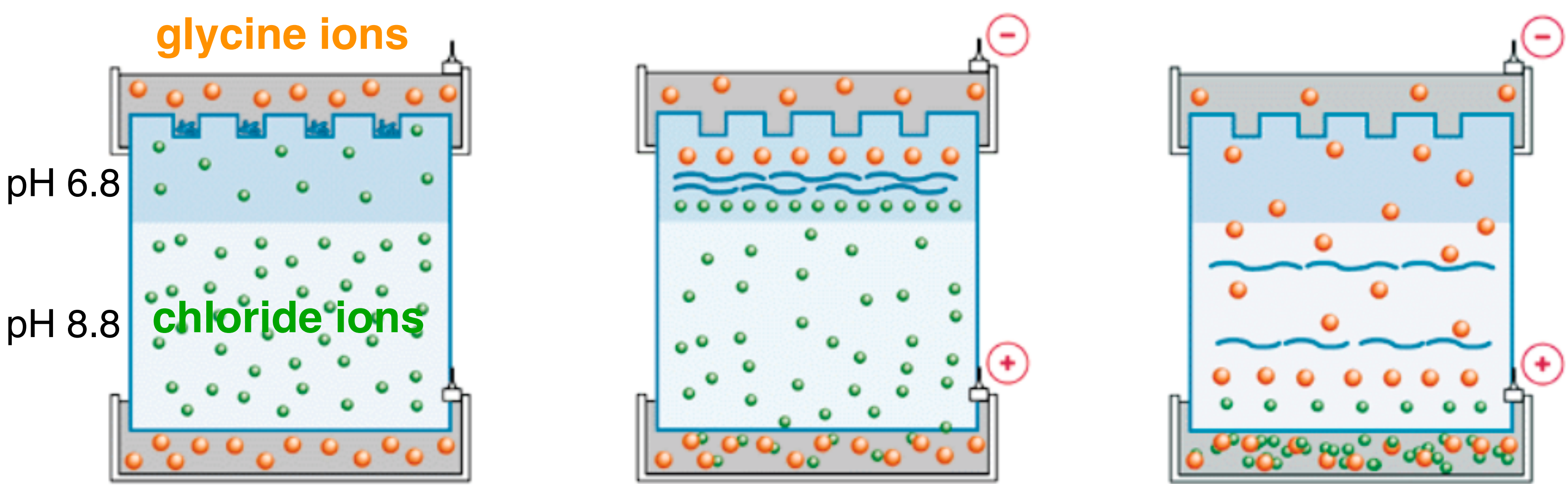
ε_{280} is the extinction coefficient; it can be determined rigorously, or estimated:

$$\varepsilon_{280} \sim n_W \times 5500 + n_Y \times 1490 + n_C \times 125$$

Assessing proteins for identity and purity

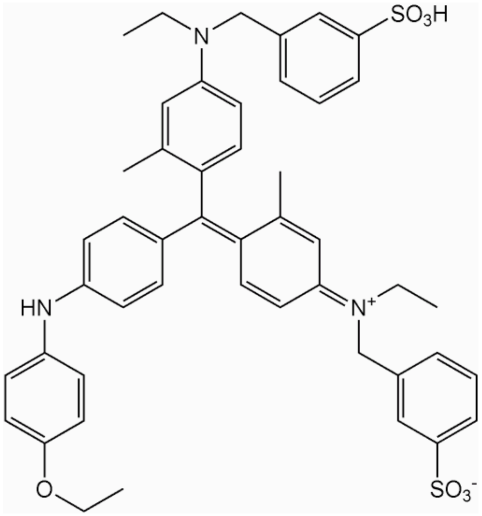
Most standard technique is sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE):

- basis is the tendency of proteins to unfold in SDS and bind a fixed amount SDS per protein (1.4 g/g)
- negative charge of SDS overwhelms protein charges
- proteins have same charge to mass ratio, but are differentially retarded by the separation gel
- stacking layer “focuses” proteins before separation layer

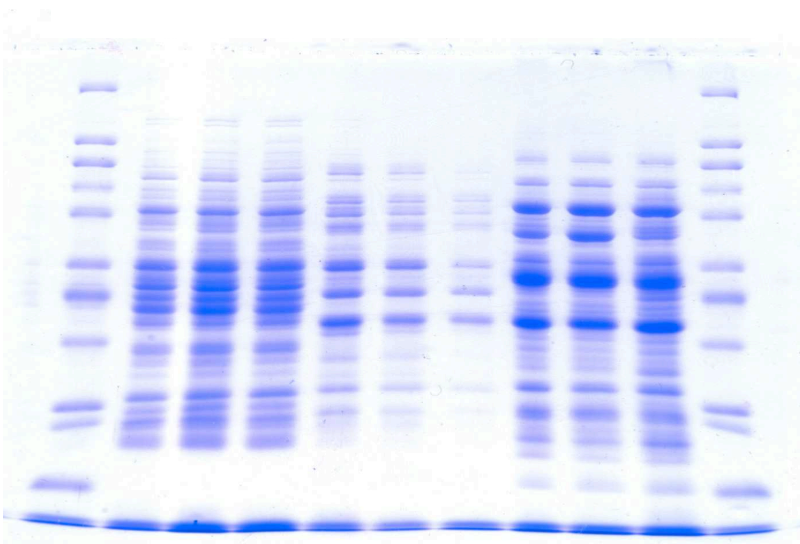


http://nationaldiagnostics.com/article_info.php/articles_id/10

Coomassie brilliant blue staining



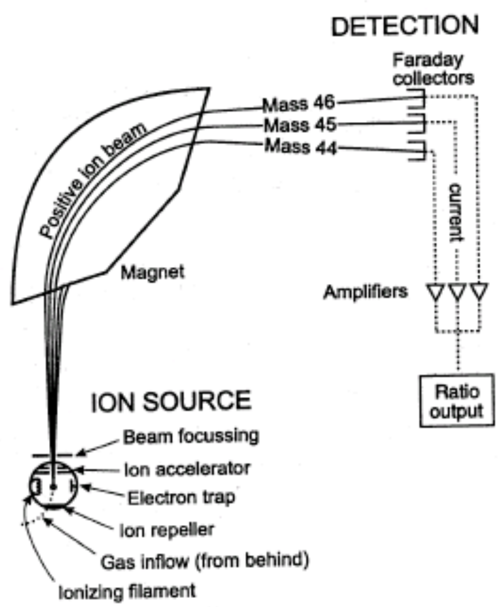
- binds proteins primarily via aromatic residues and arginine
- undergoes absorbance shift from 465 nm (brownish) to 595 nm (blue)
- basis for Bradford Assay; can be used to quantify proteins over ~3 kD

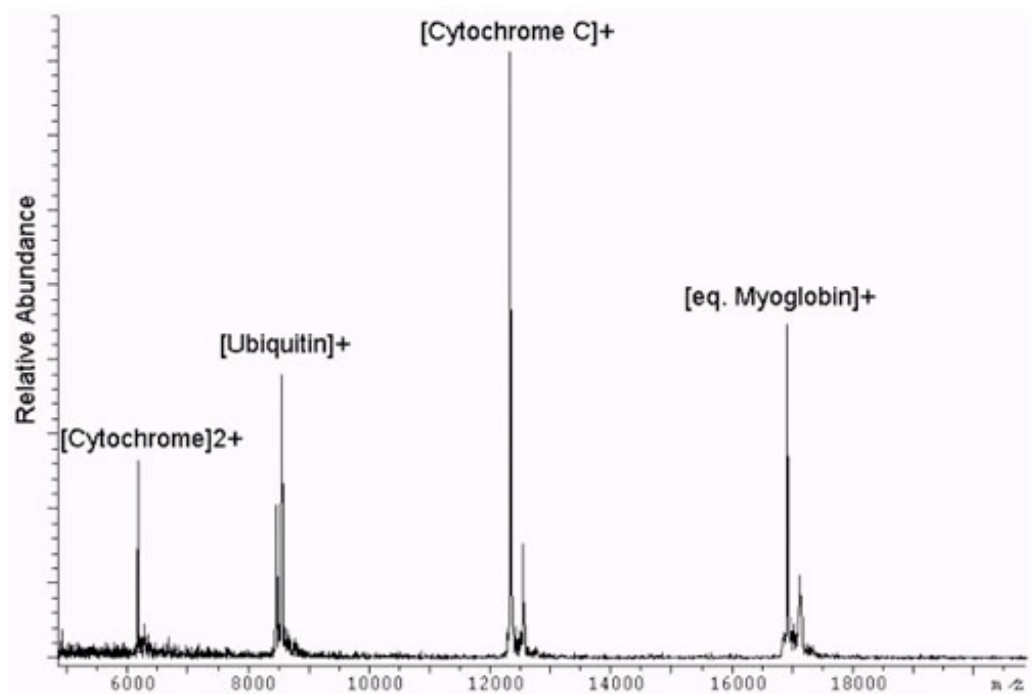


www.eiroforum.org/press/media_embl.html

SDS-PAGE gives an approximate MW and purity estimate, but how can we be sure the protein we've purified is the correct one?

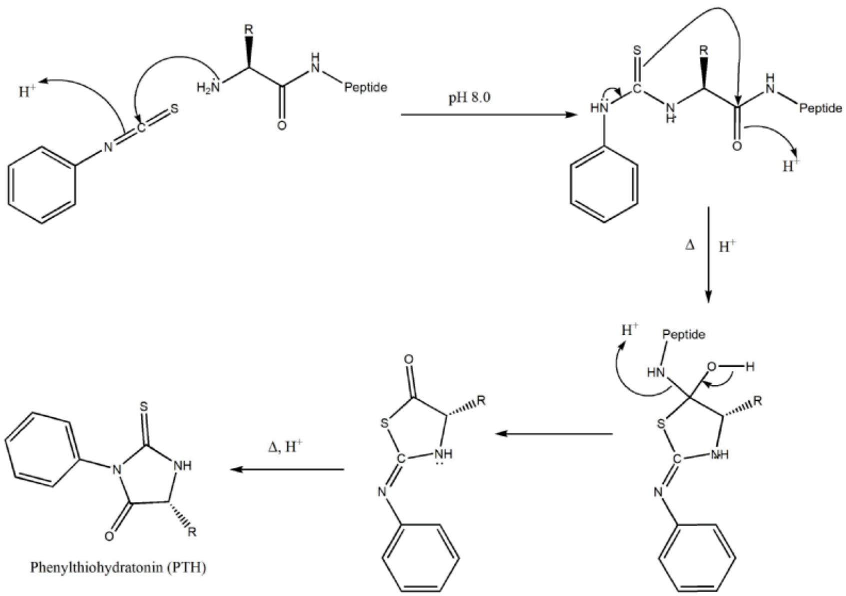
- activity assay if one is available
- knowledge of exact mass (mass spectrometry)
- N-term. sequencing and AA analysis, if necessary





en.wikipedia.org/wiki/Mass_spectrometry
www.kcl.ac.uk/ms-facility/images/maldispec2.jpg

N-terminal sequencing (Edman degradation)



- products identified by chromatography or electrophoresis
- typically ~5 cycles practical for routine N-term. sequencing

en.wikipedia.org/wiki/Edman_degradation

Amino acid analysis

- HCl digestion to digest peptide bonds
- HPLC to quantify AA components