Lecture 8:

Biomolecule Production and Purification

Question 1: True of False

Indicate if the below statements are True or False, and provide explanation where applicable:

a) Chemical synthesis methods, such as solid-phase synthesis, generally yield peptides with natural post-translational modifications like glycosylation and disulfide bonds.

b) When purifying intracellular proteins that contain free cysteine residues, reducing agents such as DTT or β-mercaptoethanol are added to prevent oxidation and unwanted disulfide bond formation.

c) Moderate salt concentrations in purification buffers can improve protein stability by shielding surface charges and reducing nonspecific aggregation, thereby improving solubility.

d) Mammalian expression systems are often used instead of bacterial systems when proper protein folding and post-translational modifications are required.

e) In affinity chromatography, the target protein binds specifically to a ligand attached to the stationary phase, and is typically eluted by adding a competing molecule.

f) In size-exclusion chromatography, smaller proteins elute before larger ones because they enter more of the pores in the resin beads.

g) In cation exchange chromatography, proteins with a net negative charge at the buffer pH will bind strongly to the column.

Question 2: Select an optimal production system for biomolecules

Many biomolecules can be obtained by **chemical synthesis, enzymatic synthesis, or cell-based expression**. For each of the examples below, select the most suitable production approach and briefly justify your choice (~2–3 sentences).

a) Carbohydrate - Branched oligosaccharide for vaccine application

b) Nucleic Acid - 25-mer silencing RNA Duplex

c) Protein - Antibody (multiple chains, ~1400 amino acids + glycosylation + disulfides)

d) Lipid - Phosphatidylcholine (PC) with Controlled Fatty Acid Composition

Question 3: Select an optimal expression system for your protein

What expression systems would you choose for the expression of the following proteins? Why or why not and explain:

a) 12 kDa single-domain protein of bacterial origin, residing in cytoplasm (example: KaiB oscillator protein)

b) 280 kDa multi-domain protein of human origin, residing in cytoplasm (example: mTOR kinase)

c) 15 kDa disulfide-linked chemokine of mouse origin, secreted outside the cell (example: Interleukin 11)

d) 249 kDa multidomain, N-glycosylated, transmembrane protein of human origin (example: integrin)

e) 194 MDa virus of bacterial origin (example: T4 bacteriophage)

Question 4: Cell culture contamination

When working with mammalian cells (such as CHO, HEK), the work is always conducted in sterile environments (biological safety cabinets) to avoid any contamination with bacteria or yeast.

a) Why is bacterial contamination such a big problem when growing mammalian cells? Discuss as many potential issues as you can think of.

b) Imagine that you were growing CHO (Chinese Hamster Ovary) cells in a 2 L bioreactor. The cell density is 1 x 10^6 cells/ml and you just provided them with fresh media for optimal growth. Accidentally, you introduce 1 bacterial cell (E coli) into this cell culture. Assuming the doubling rates of 24 hours for CHO cells and 0.5h for bacterial cells under these conditions, estimate the length of time that will take bacteria to match the total concentration of CHO cells in this flask?

Question 5: Select optimal columns for biomolecule purification

What columns or chromatography techniques would you need to complete the following biomolecule purification experiments:

a. Separate two proteins based on differing molecular weights

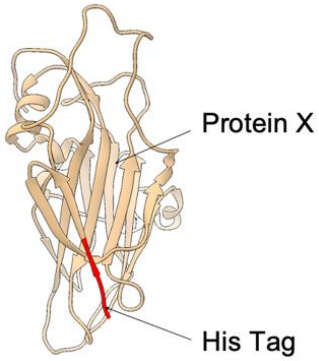
b. Separate a mixture of lipids based on differing hydrophobic properties

c. Separate a mixture of DNA molecules that have different lengths and resulting negative charge (e.g., 20, 40 and 60 nucleotide base pairs)

d. Purify a strep-tagged protein from other cellular biomolecules

Question 6: Protein X purification

You would like to purify a human intracellular protein (Protein X) which has a molecular weight of 40 kDa and is associated to several diseases. Based on sequence analysis and AlphaFold structure prediction, you conclude that it consists of a single domain and is not expected to undergo any post-translational modifications. Further, the protein does not have any cysteine residues that can form disulfide bridges. You added a poly-histidine tag to the C-terminus of the protein to facilitate purification.



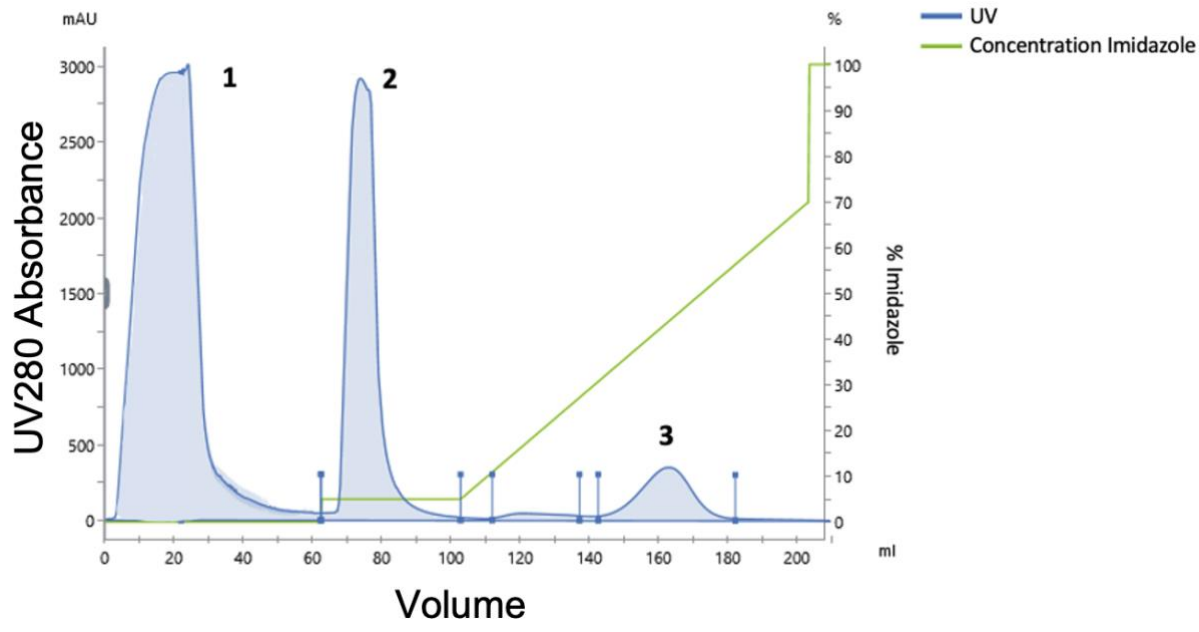
a. Which expression system would you first test for this protein and why?

b. Following the expression in the host chosen previously, you continue with affinity chromatography in order to purify the protein. Describe the specific type of affinity purification you'll employ and identify which part of the protein is required for this method. Additionally, explain the working principle of this technique and specify the type of column you'll be using.

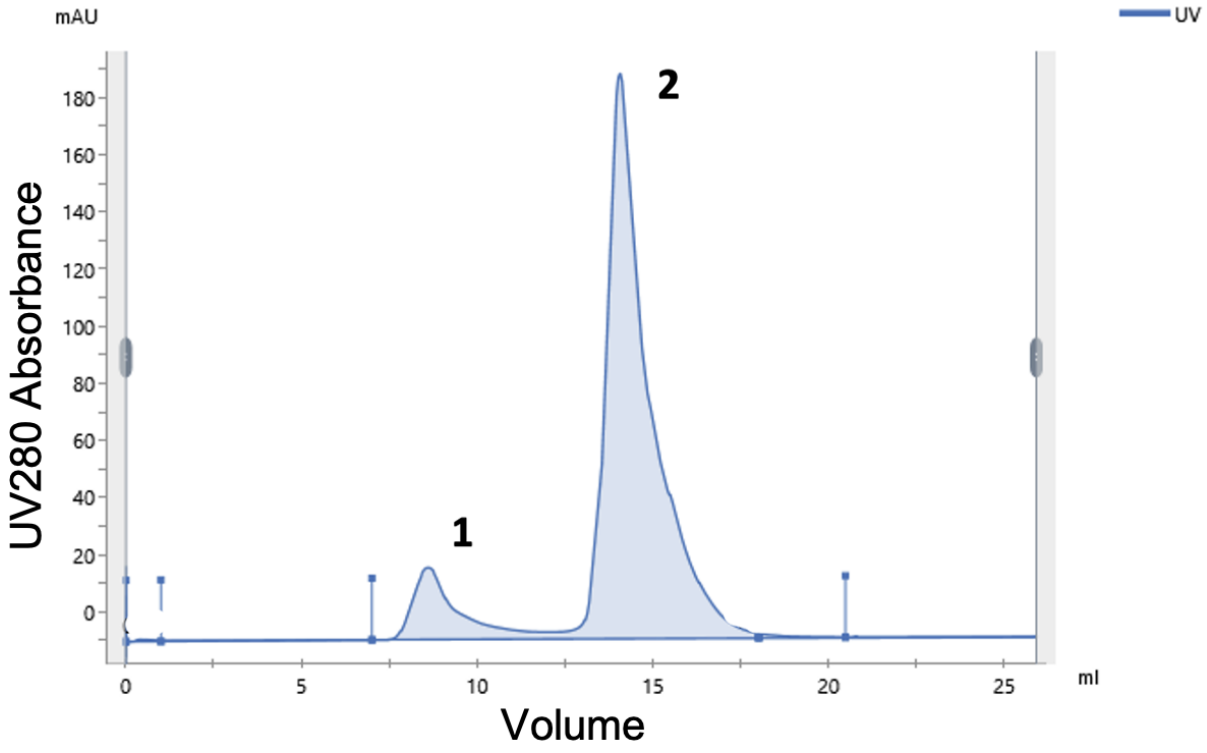
c. Select the minimal set of necessary buffer components that should be included in the Elution Buffer for the his-tag purification step?

Reagent	**Yes**	**No**	**Why?**
20mM Tris-HCl			
0.1% Triton-X			
150mM NaCl			
5mM DTT			
10mM Ribose			
20% Glycerol			
0.1mM Imidazole			
500mM Imidazole			

d) For the His-Purification you obtain the following chromatogram. Label the peaks 1, 2 and 3 and explain what proteins are in each peak. In blue you can follow the UV absorbance of the protein at 280 nm and in green the concentration of imidazole.



e) You suspect that your protein of interest may be partially aggregated after the affinity purification. This could lead to problems with downstream structural and functional analyses. So you decide to separate the monomeric and aggregated forms of the protein using Size-Exclusion-Chromatography. How does size-exclusion chromatography work? Looking at the chromatogram, where is the aggregated and where is the soluble protein?



Question 7: Isoelectric points and Ion-exchange chromatography

You are analyzing a mixture of two synthetic peptides produced by solid-phase synthesis:

Peptide A: **WDGDAKRVTSA**
Peptide B: **SEDDVKKKAGL**

Assume that the peptide is under standard aqueous conditions and the following pKa values apply:

Group	pKa
C-term (α–COO^-)	2.0
N-term (α–NH_3^+)	9.0
Aspartate side chain	4.0
Glutamate side chain	4.1
Lysine side chain	10.8
Arginine side chain	12.5

a) Define what charged groups will exist in each peptide at pH 7.

b) Based on the analysis of charged groups in a) and their charge status you conclude that both peptides will carry net neutral charge at approximately pH 7. For each peptide, determine the bracket pKa values flanking pH 7? Use the bracket pKa values to calculate the exact pI value for each peptide?

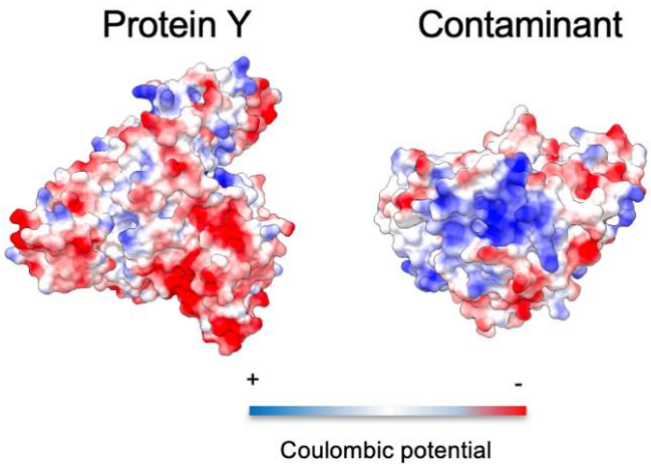
c) What would be the net charge of each peptide at pH 3.0? Hint: Use lecture 5 to help you determine what happens with each charged groups at different pH.

d) If you wanted to immobilize the peptides to an ion-exchange column at pH 3.0, what would be the prefered type of column?

e) You proceeded with peptide immobilization at pH 3.0 using the selected column. Now both peptides are bound and you are trying to select an elution strategy that would allow to separate them. The decision is between using either (i) pH or (ii) salt gradient. Which approach would allow you to separate these peptides into distinct peaks? In both cases, discuss the order at which the proteins will elute.

Question 8: Separating proteins by charge

You are working with a Protein Y with a molecular weight of 50 kDa that has a pI of 5.5. After the initial affinity purification you realize that the protein is contaminated with other protein species, so you decide to increase the purity of your protein by separating it based on charge, using ion-exchange chromatography.



a) Using a buffer of ph=7.5 what is the net charge of the protein? What subtype of column would you use to bind it?

b) What are the different ways that you can use elute proteins from this type of column?

c) You elute the protein from the column using a linear gradient of NaCl and get the following chromatogram. In the chromatogram you follow the protein by looking at its absorption at 280 nm (UV). By increasing the relative percentage of the "elution buffer" (green) you slowly increase the salt (NaCl) concentration loaded onto the column which triggers the elution of the protein. You can follow the increase in salt concentration by looking at the increase in conductivity (orange). You observe that the contaminating proteins elute before your protein of interest. Why is that the case? What does the position of the peak depend on when eluting with salt?