BE 167L - Bioengineering Laboratory

Lab 3: Bioconjugation

Prelab reading

Bioconjugation is the creation a covalent bond between molecules, at least one of which is a biological molecule. You will be conjugating a small fluorescent molecule (fluorescein) to a large protein (bovine serum albumin, BSA). Bioconjugate chemistry is useful to the bioengineer because it can allow tagging of biological molecules with something that is easily tracked by different instruments. Things that might usually be difficult to observe or measure (such as location and concentration of specific proteins in a living cell) can become visible under a microscope if you tag it with something that glows or fluoresces. Joining biological molecules to each other (or to chemical molecules that have special functions) can help a researcher study functions of enzymes in different environments or create new biological molecules or cells that may have clinical applications.

There are many different chemical reactions that are commonly useful when conjugating biological molecules because most of these molecules are composed of similar elements, such as amino acids or sugars. These reactions have been discussed in lecture and represent a tool-box of relatively well-understood mechanisms of forming covalent bonds between specific types of chemical functional groups. Consider them different routes suggested by a GPS for getting from point A (two separate molecules) to point B (covalently joined molecules). There may be other ways, but these are the most common and useful. You will use NHS-fluorescein (N-hydroxysuccinimide attached to a fluorescein molecule) which is quickly and easily conjugated to proteins that contain primary amines (like lysine) in the amino acid sequence, provided that the lysines are accessible in the protein's folded state. Refer to your lecture notes for the mechanism of the reaction.

The reaction takes place at room temperature just by mixing the molecules together. Because the NHS is a good leaving group it is easily displaced by the amine groups on a protein once they contact each other. You just need to provide the right conditions. Having more NHS-fluorescein than protein ensures that there are enough to easily bump into the available amines on the proteins. Having a reaction buffer (PBS, pH 7.4) which is just slightly basic helps to keep the molecules and reactive groups in the right folded conformation and protonation state for the reaction. One thing to be careful of is to bring the NHS-fluorescein in contact (mix it) with the protein before it reacts with water in the air. Being a good leaving group also makes NHS unstable. NHS-fluorescein is stored in the freezer as a dry powder. Your TA will dissolve it in N,N Dimethylformamide (DMF) which is an organic solvent with little to no water. The NHS should not contact water until it is mixed directly with your protein solution (DMF is miscible with water). To increase the probability of the NHS reacting with your protein rather than the water you should have a relatively high concentration of protein. These ratios and concentrations will be determined for you in this lab, but for future experiments keep these caveats in mind.

Once sufficient time has passed for the reaction to take place you will purify the fluorescein-BSA conjugate from unreacted and excess NHS and fluorescein. Sephadex is a white powder which is very porous. You will use it to make a small gel filtration column. It has been soaked in PBS, the same solution as your reaction buffer. These particles, when packed together in a tube, can filter out small molecular weight molecules. Think of them as beads that have tiny pores. A tube of packed beads allows small things into its pores base on size, trapping them for a while. Larger molecules pass right by the beads. The sephadex you will use has a specific pore size that traps free fluorescein and NHS, while large molecules, like your protein, pass through the column. Of course if you flush the column long enough with a lot of liquid, eventually everything will come out.

The degree of labeling on your BSA (how many fluorescent molecules you have on each protein molecule) will affect how strongly fluorescent your protein will be under the microscope. It will also affect the protein's solubility. Moreover, it is

an indicator of how well the reaction went. You can calculate the degree of labeling by measuring the absorbance of the solution at two different wavelengths. Proteins most efficiently absorb light with a wavelength of 280*nm*. Fluorescein absorbs most at 494 nm. Measuring the absorbance at these two wavelengths can give you the relative concentrations of protein to fluorescein in your solution, scaled by their molar absorptivity or extinction coefficient. The equations for this calculation are given to you in the procedures below. If you look up the structure of your protein, you can identify how many sites on the protein are potentially able to react with the NHS-fluorescein, for example 10. If the reaction was 100% efficient, you should calculate 10 fluorescein molecules per protein. You will likely have a lower ratio, but hopefully more than 1, meaning you have at least 1 fluorescein per protein and all your proteins are labeled.

Watch the primer video.

Optional pre-lab readings:

Highly Recommended: Stephanopoulos, N., & Francis, M. B. (2011). Choosing an effective protein bioconjugation strategy. *Nature Chemical Biology*, *7*(12), 876–884. doi:10.1038/nchembio.720

Recommended: Farkaš, P., & Bystrický, S. (2010). Chemical conjugation of biomacromolecules: A mini-review. *Chemical Papers*, *64*(6), 683–695. doi:10.2478/s11696-010-0057-z

Pre-lab assignment:

See separate document.

Preparation

Reagents

- PBS, pH 7.4
- Bovine Serum Albumin (BSA), powder form, 66,776 Da
- NHS-fluorescein dissolved in DMF (1 mg/100 µL)
- Sephadex soaked in PBS

Supplies

- 2 scintillation vials
- 2 long glass pasteur pipettes a small pinch of cotton
- 1 plastic cuvette

Equipment

- Pipettes and tips
- Scale
- UV-Vis spectrophotometer (Biomate 3S)

Safety

In addition to your usual precautions, be aware that the NHS-fluorescein is dissolved in N,N-Dimethylformamide (DMF). While you will only be handling small amounts and diluting in water, it is still an organic solvent and a possible carcinogen. Take care when handling anything containing your NHS-fluorescein and dispose of your waste properly, through chemical waste containers.

Procedure

1. Make 2 mL of a 5 mg/mL solution of BSA in PBS using a glass scintillation vial.
2. Based on the molecular weight of BSA and NHS-Fluorescein, calculate the volume you will need from a 1 mg/100 µL solution of NHS-fluorescein in order to have 7 moles of NHS-fluorescein per mol of BSA. Start by calculating the number of moles of BSA you just dissolved.
3. BSA MW = 66,776 Da = 66,776 g/mol NHS-Fluorescein MW = 473.4 g/mol
4. Add the proper volume of NHS-fluorescein into your vial of BSA.
5. Cap it, wrap with foil and label with your initials. Place it on a shaker for 1 hour at room temperature.
6. You want to protect the fluorescein from too much light exposure to prevent photobleaching. Note: during this waiting period you may prepare you sephadex column.
7. Prepare a column for purifying your conjugated product. Take a small pinch of cotton between your fingers (like 1 of the size of a Qtip) and make a loose ball. Place it in the top of one of your pasteur pipettes. Use the other pasteur pipette to push that ball down into the narrowing of the pipette until it plugs the pipette tightly.
8. Gently pipette about 500µL of the sephadex suspension into your plugged pipette. If particles get stuck to the sides high up rather than settling nicely to the bottom, rinse the walls with just PBS. Allow the PBS to drain from the column into a waste container either by gravity, or by GENTLY applying pressure from a plastic bulb. Stop when there is just 1mm of PBS above the surface of the sephadex.
9. Place your sephadex column into a clean glass scintillation vial. You may have to take turns holding it upright. Transfer your protein-fluorescein solution from the first glass vial into the sephadex column, being careful not to disturb the sephadex pack in the pipette. Collect the filtrate in your new glass vial. You may wait for gravity to pull it through, or GENTLY push it through with pressure from a plastic bulb. You will need to filter your entire solution in a few batches, but you can use the same column. You don't need to flush with any PBS at the end, just push the remainder through.
10. You will now quantify the degree of labeling on your protein. Your TA will prepare a blank cuvette for the class to share, containing just 1mL of PBS. In your own cuvette, dispense 990 µL of PBS and 10 µL of your purified protein-conjugate filtrate. Mix well. Label the top edge of your cuvette with your initials.
11. Place your cuvette into the UV Spec, remember your place number.
12. Your TA will coordinate reading 5 cuvettes at a time in the cell changer. Record the Absorbance (abs) of your sample at 280 nm and 494 nm. Make sure the UV Spec is blanked at each wavelength. You want your abs for both wavelengths to fall between 0.1 and 1.5. If your readings fall outside that range, prepare a new solution with a slightly higher or lower concentration depending on if you need your readings to be higher or lower. Do this separate from your original filtrate in a different tube. Make a note of your dilution factor. Assuming you have not significantly changed the dilution of your original 5 mg/mL solution of BSA, what is the estimated molar concentration of your protein conjugate in the filtrate? Is this more likely to be an overestimate or under-estimate? Why?
13. Store your filtrate wrapped in foil in the refrigerator until the next lab. Make sure your initials are on it.
14. Using the equations below, calculate the degree of labeling of your fluorescein-BSA.

$\boldsymbol{\varepsilon}$ = Molar extinction coefficient

Molar extinction coefficient of BSA: $\boldsymbol{\varepsilon}_{BSA} = 43{,}824 M^{-1}cm^{-1}$

Molar extinction coefficient of Fluorescein: $\boldsymbol{\varepsilon}_{Fl} = 70{,}000\ M^{-1}cm^{-1}$

$A_{max} = A_{494}$ for this experiment

CF = Correction factor = 0.30 for this experiment (CF of plain fluorescein)

$A^{280} - (A^{max} \times CF)$

Protein Concentration (M) =

$\sum BSA$

A^{max} of labeled protein

Mol of fluorescein per mol of protein =

$\sum_{Fl} \times$ protein concentration (M)