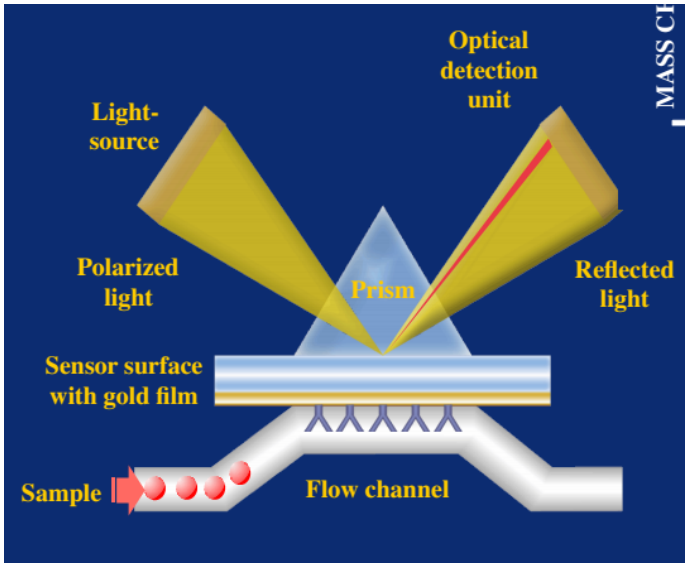
BMB/Bi/Ch 173 – Winter 2017
Problem Set 8.1: CD and SPR– Assigned 2-28-17. Due 3-7-17 by 10:30am
100 points total

1. **(25 points) SPR Basics**
    a. (10 points) Draw a typical schematic of a Biacore SPR instrument and explain how signal is measured to produce affinity data for a biomolecular interaction.



In SPR, a wedge of polarized light is focused on a conductive slide (typically made of gold) under the conditions of total internal reflection. At a characteristic angle of light, energy transfer occurs between the light wave and the surface plasmons, resulting in a thin shadow in the reflected wedge of light. This angle depends on the refractive index of the material, which in turn depends on the mass of the film. When an analyte binds to a ligands on the SPR chip, the mass difference leads to a change in the angle of maximum light absorbance, which is the signal measured by the instrument.

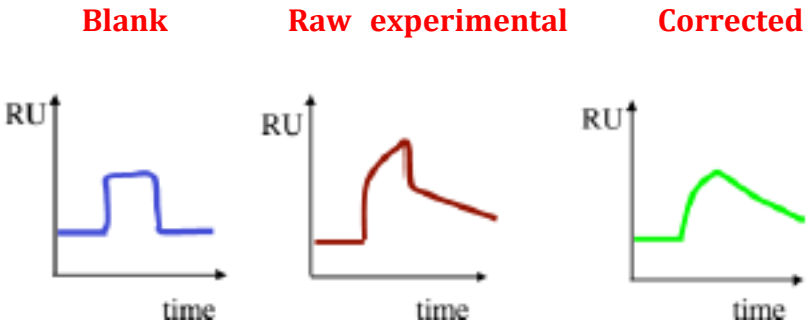
    b. (6 points) Why do SPR experiments include a surface regeneration step?

In a typical SPR experiment, different concentrations of analyte are flowed over the surface of the chip with the ligand. A regeneration step is necessary to remove all of the bound analyte so that the surface can be reused for the next analysis.

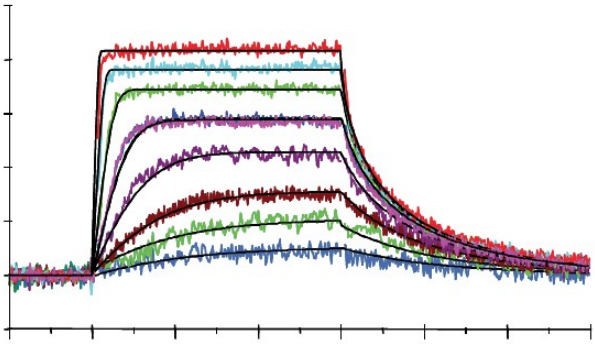
    a. (9 points) Given the raw experimental sensorgram shown below, you noticed that it didn't look right because of the sudden jumps in the curve (pointed by the arrows). Explain why and how to correct for it using the concept of "bulk refractive index".

The SPR signal depends on changes in the refractive index at the surface of this chip, and the refractive index changes when the buffer composition changes. Therefore, the addition of analyte buffer will often cause a signal change regardless of whether or not analyte binds to the surface, producing a characteristic step in the curves. To correct for this in an SPR experiment, buffer + analyte is flowed over a chip that does not contain immobilized ligand. This "blank" run is subtracted from

the experimental results to correct for the bulk changes in refractive index.



2. **(30 points) Analyzing SPR Data:** You carry out an SPR experiment with the growth factor and its antagonist to produce the sensorgram below.



a. (4 points) Label the axes with appropriate units.

x-axis: time y-axis: response units

b. (6 points) There are three distinct experimental phases in this sensorgram. Label each phase and explain what is happening during that part of the experiment.

(1) Baseline: buffer is flowing over the chip
(2) Injection: analyte is flowing over the chip. During this phase, analyte is binding to the ligand on the chip, causing an increase in signal. At the same time, ligand is also dissociating, and eventually equilibrium is reached (signal plateau).
(3) Dissociation: buffer is flowing over the chip. Analyte is dissociating from the ligand on the chip, causing a decrease in signal.

c. (8 points) What rate constants contribute to the curve at each of the phases in part (b)? How can the association and dissociation constants (k_a and k_d) be determined?

(1) Baseline: none
(2) Injection: While k_a dominates at the beginning, the shape of this curve is

determined by both k_a and k_d (which eventually reach equilibrium)
(3) Dissociation: only k_d
kd can be determined by fitting the dissociation phase to an exponential decay. ka and kd can be determined by fitting the association phase to the slightly more complicated exponential equation below. Biacore evaluation software can do global fits of both phases simultaneously.

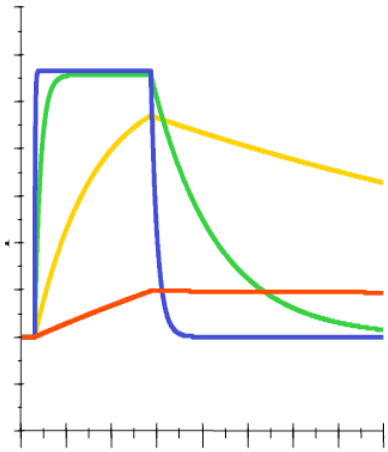
$$Dissociation: \; R = e^{-k_d t}$$

$$Association: \quad R = R_{max}\frac{k_a C}{k_{obs}}(1 - e^{-k_{obs}t}) \quad with\ k_{obs} = k_a C + k_d$$

d. (5 points) How do you determine the binding affinity K_d? What needs to be true about the interaction in order to attain this constant?

Using the association and dissociation constants, you can get the binding affinity of the analyte to the ligand. Kd = kd/ka. In order for you to be able to get th is measurement, you must know that the reaction is 1:1.

e. (7 points) You move on to screening the library of small molecule inhibitors of the growth factor. Four molecules look promising, producing the four curves shown below. All four molecules have the same equilibrium dissociation constant K_d and were used at the same concentration but display very different SPR curves. Explain this observation.



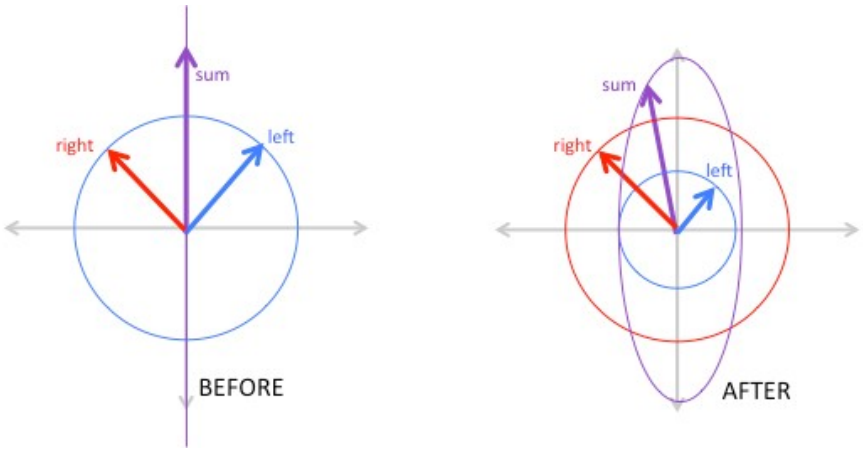
While the four small molecules have the same K_d, they have VERY different binding kinetics – aka different association and dissociation constants (k_a and k_d). Since $K_d = k_d / k_a$, a molecule with a fast on-rate and a fast off-rate can have the same K_d as a molecule with a slow on-rate and a slow off-rate. In this case the molecules rank as follow:
k_a: red < yellow < green < blue
k_d: red < yellow < green < blue

3. **(27 points) CD Basics**
   a. (7 points) What is the difference between CD and circular birefringence?

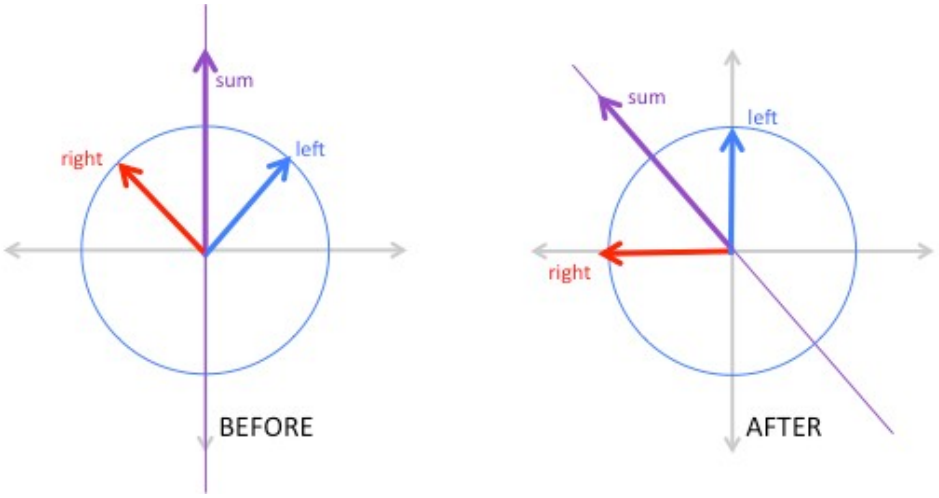
      CD occurs when a material absorbs right--- and left---handed circularly polarized light differently. Circular birefringence occurs when the refractive indices for right ---and left---handed circularly polarized light differ.

   b. (10 points) If you shine linearly polarized light at a circularly dichroic material, what would be its polarization as it emerged? What if the material were circularly birefringent? Why? (Diagrams maybe helpful)

      For circularly dichroic material, the light would change from linearly polarized to elliptically polarized due to the differential absorbance:



      For circularly birefringent material, the light would remain linearly polarized but emerge rotated with respect to the original beam due to the phase shifts:



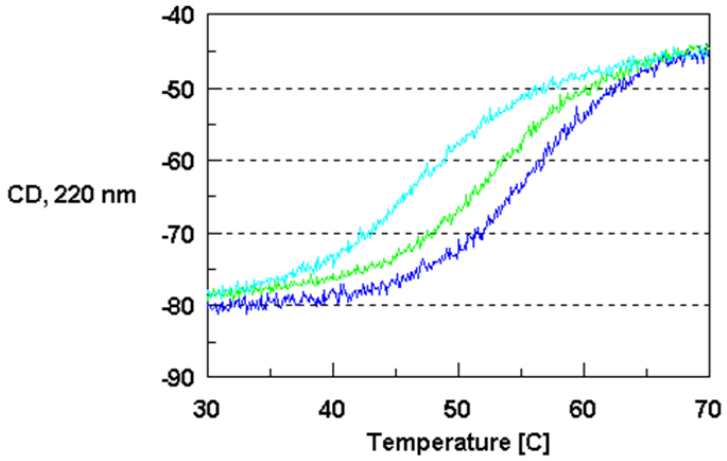
c. (10 points) What information does a CD spectrum provide? What are the advantages and disadvantages of CD in comparison to other structural techniques such as NMR or crystallography?

A CD spectrum provides general information about the secondary structure of a protein – ie whether alpha helices or beta sheets are present. In comparison to other structural techniques, CD requires small amounts of material (both small volumes and low concentrations) and has quick and easy data collection. However, CD is limited because it only provides general secondary structure information, so small perturbations will not be detected. Similarly, CD does not provide information about tertiary or quaternary structure. Therefore, the major disadvantage to CD in comparison to other techniques is the lack of detailed structural information.

4. **(20 points) Using CD in the lab:** You work at a biotech company that has just discovered a protein that can be used as a protein therapeutic.
   a. (6 Points) The protein originates from a fungus, but your company wants to mass produce the protein in mammalian cells. How can CD be used to compare the native and recombinant proteins?

      Comparing the CD spectra of the native and recombinant proteins will show whether or not they differ in overall secondary structure. If the two spectra align well, then the two proteins have similar structure. If not, the move to the recombinant system has altered the protein folding.
   b. (6 points) You suspected that your recombinant protein has altered folding than the native one. To thoroughly compare the folding and folding kinetics of the native and recombinant proteins using CD, what variables might you test? (hint: factors affect folding kinetics)

      Temperature, protein concentration, denaturant concentration, pH
   c. (8 points) You want to increase the shelf---life of your product, so you examine the stability of the protein in different buffers using CD. You produce the data below, where each curve represents a different buffer:



- (3 points) Describe the experiment.

The protein was put into three different buffers. The CD at 220nm was measured at increasing temperatures as a readout for the presence of folded protein, generating a melting curve for the protein.

- (2 points) What information does the CD measurement at 220 nm provide?

The signal of both alpha helices and beta sheets appear strongly in the 220nm region. The molar ellipticity at this wavelength will provide a readout of the general structure and stability of the protein.

- (3 points) Which buffer is best for the protein?

The dark blue curve retains secondary structure high temperatures better than the other two curves, indicating that the protein is the most stable in this buffer