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BIOE111: Functional Biomaterial Development and Characterization
FINAL EXAM (April 27, 2017)

Question 0: Fill in your name and student ID on each page. (1)
Question 1: Briefly define the following terms (15):

a) Tandem Mass Spec (**3 points**)

A two-step mass spectrometry method. First a sample is ionized and separated by mass-to-charge ratio (m/z). Ions of a particular m/z generated in the first step are then fragmented in the second step, resulting in a new spectrum of ions. The characteristic fragmentation pattern from the parent ion provides structural information about a peptide. Tandem mass spectrometry can be used to sequence and identify unknown peptides and for proteomic analysis.

+1 Two steps: ionization then fragmentation
+1 separate ions by m/z
+1 application

b) AFM (**3 points**)

Atomic force microscopy is a type of scanning probe microscopy that uses a cantilever with an extremely fine tip to characterize surfaces with up to near atomic resolution. AFM can be used for both topographical imaging and mechanical characterizations such as stiffness or force measurements.

+1 Scanning probe microscope
+1 sharply tipped cantilever interacts with surface of material
+1 application

c) Shotgun DNA sequencing (**3 points**)

A method of sequencing long strands of DNA. DNA is digested into smaller fragments and cloned into viral vectors. Insertion of the fragments into a vector allows for the use of a universal primer set to sequence the forward and reverse strands. The vectors are sequenced (by Sanger sequencing) and the reads are aligned to form a contiguous sequence of the original DNA strand.

+1 digested into small fragments
+1 cloned into vector and sequenced
+1 used for large strands of DNA

d) ITC (**3 points**)

Isothermal titration calorimetry is a technique that can be used to determine binding affinity, stoichiometry, and the thermodynamic parameters associated with a binding

interaction or chemical reaction. A ligand is titrated into the sample cell where it interacts with its counterpart causing heat to be released or absorbed. A heater is powered on either the sample cell or the reference cell in order to maintain both at the same temperature. The energy added to the system is known and used to characterize the binding interaction or reaction.

+1 used for affinity, stoichiometry, thermodynamics
+1 titrate ligand
+1 maintain reference cell and sample cell at the same temperature

e) FRET (**3 points**)

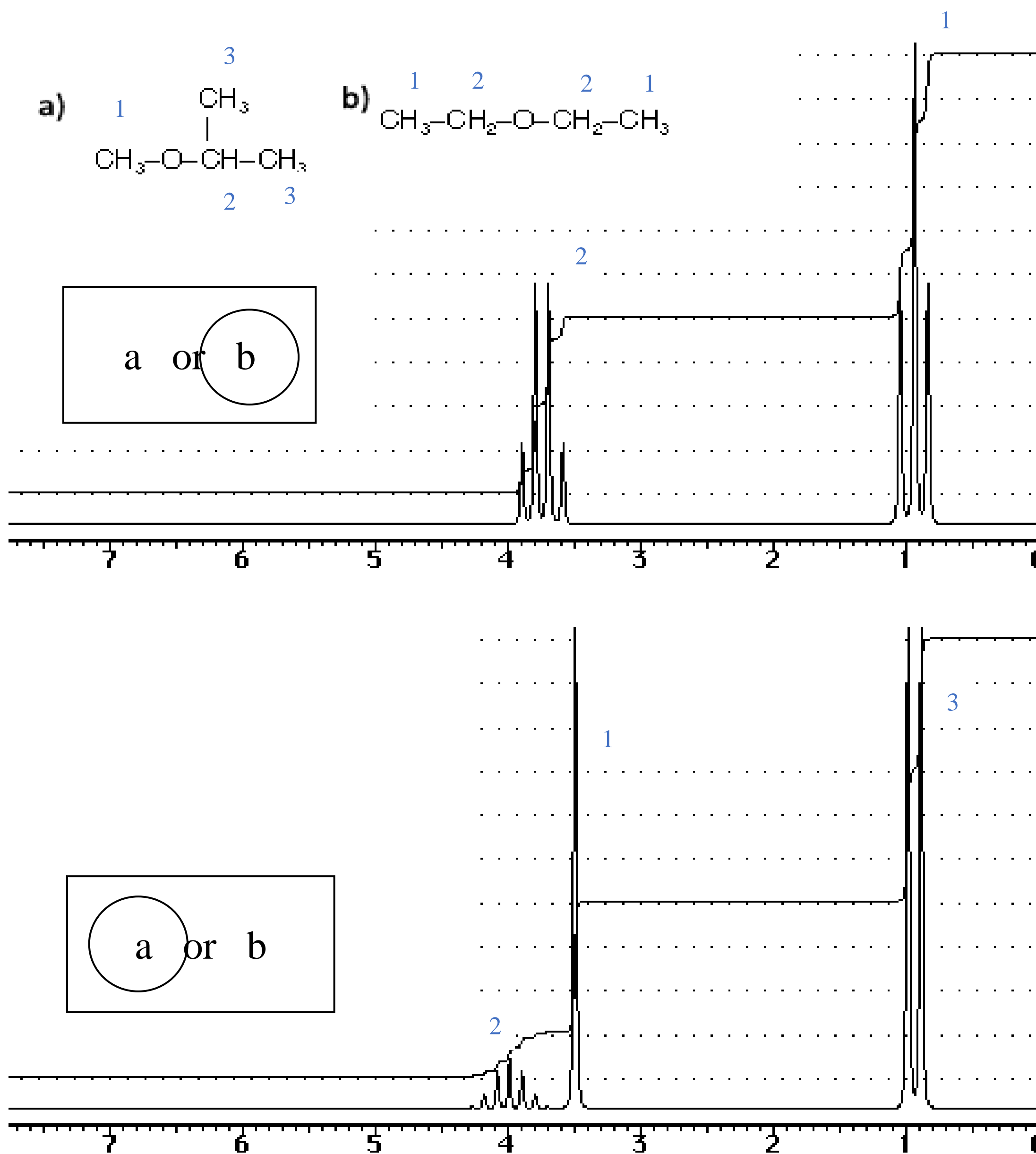
FRET is a physical phenomenon called fluorescence resonance energy transfer. FRET occurs when a donor fluorophore is excited and transfers its energy to a nearby acceptor fluorophore, which then emits light as the electron relaxes to its ground state. FRET is extremely sensitive to the distance between the donor and acceptor fluorophores.

+1 two fluorescence doner and accepter
+1 energy transfer between fluorophores
+1 distance sensitivity

Name with Last Name, First: **Student ID:**

Question 2. NMR (Total 15 points)

a) Structures a and b were analyzed by H-NMR. Circle the letter corresponding to the molecule that is associated with which spectrum. Assign all protons to their appropriate peak on each spectrum. (**6 points**)



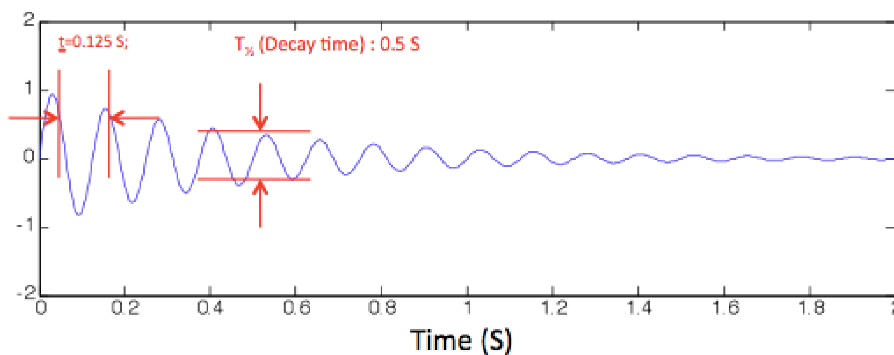
Name with Last Name, First: **Student ID:**

b) Suppose that we will apply an additional field on the chemical sample in the second NMR spectrum in part a with the signal corresponding to 3.4 ppm to saturate all the corresponding protons to excited states. How will the NMR spectrum change and explain why **(5 points)**.

This is called the Nuclear Overhauser Effect. The peak at 3.4 ppm will disappear because all of the nuclear spins of the corresponding protons will be saturated and no switching will be occurring. The nuclear spin polarization of these protons can be transferred to other populations via cross-relaxation resulting in a reduction in peak intensities. The intensity of the peak around 4 ppm will be reduced slightly because the corresponding protons are less than six angstroms from those forming the peak at 3.4 ppm. The protons forming the peak at 1 ppm are farther than six angstroms away, so the intensity of this peak will not change.

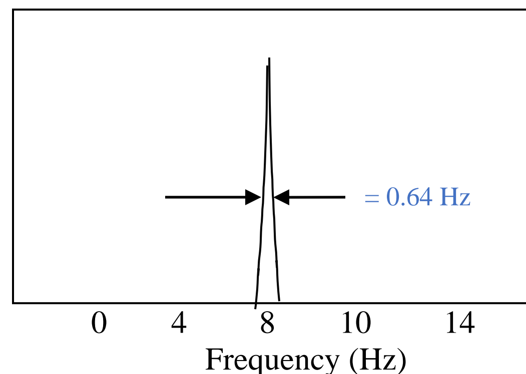
+1 Identify Nuclear Overhauser Effect
+1 Occurs due to transfer of spin polarization
+3 Correct description of changing peak intensities

c) Convert the following time-dependent signal into the frequency domain (Fourier transform) and sketch the frequency graph below. Label the frequency axis with units. Determine the line broadening by solving for the width at half-height of any peaks **(4 points).**



Width at half height
$\Delta v1/2 = 1/(\pi T1/2) = 1/(\pi * 0.5) = 0.64$ Hz

+2 correct math
+2 correct graph



Name with Last Name, First: **Student ID:**

Question 3 DNA Sequencing (20 points)

5’ATCGCAACGG**AAAAGTTATT**AACAATATGT**TACGCAGCGA**GGAAGTAAAC3’
3’TAGCGTTGCC**TTTTCAATAA**TTGTTATACA**ATGCGTCGCT**CCTTCATTTG5’

Suppose that we will sequence part of the DNA shown above using Sanger DNA sequencing methods. We will use the primer **5’ AACATAT 3’** for DNA sequencing. You have a tube containing the DNA template, primer, billions of single A C G and T nucleotides and billions of **dideoxy A, C, G, and T** nucleotides.

a) After the PCR reaction for DNA sequencing, how many different DNA strand lengths will have formed? (Assume you ran the replication reaction for a long time, therefore, there are millions of each fragment). **(5 points)**

24 strands, one for each base after the primer and DNA is synthesized 5’ to 3’

+5 all correct
+3 incorrectly counted as if DNA synthesis occurs 3’ to 5’ direction giving 19 strands
+3 conceptually described correctly but miscounted. Requires description to prove full conceptual understanding for partial credit.

b) Predict the maximum length (in nucleotides) of the DNA strands you would find after PCR reaction. **(5 points)**

31 bases, 24 + 7 base primer

+5 correct
+5 if included primer but used incorrect longest strand from part a
+0 did not include primer

c) Suppose that we will sequence the above DNA using pyro DNA sequencing methods. What new chemicals or reagents are necessary to accomplish pyro DNA sequencing and briefly explain how pyro sequencing works **(5 points)**.

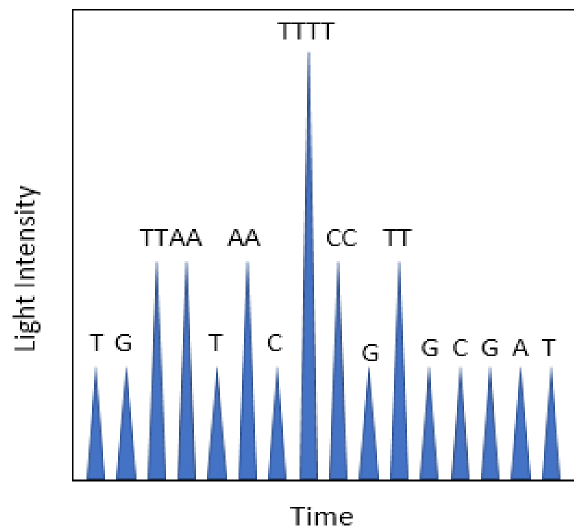
New reagents: ATP sulfurylrase (and substrate adenosine 5' phoshosulfate), luciferase (and substrate luciferin), apyrase

+0.5 each (1.5 total)

Pyrosequencing is a sequence by synthesis of DNA method. A primer anneals to the template strand to initiate the PCR reaction. All normal PCR reagents are present except only one purified dNTP is added at a time. If the correct dNTP has been added, a pyrophosphate will be released and converted into ATP by the ATP sulfurylase. The luciferase will use the ATP to generate light. The intensity of the emitted light corresponds to the number of ATP molecules generated and therefore the number of dNTPs that have incorporated into the growing DNA strand. Apyrase degrades unused dNTPs. If no light is released upon addition of the pure dNTP, this indicates it is not the correct dNTP. Another purified dNTP is added until light emission indicates the proper base pair for the template strand has been incorporated.

+1 One dNTP at a time
+1.5 Correct description of ATP sulfurylase, luciferase, and apyrase activities
+1 Light intensity corresponds to dNTP incorporation

d) What is actually measured during pyro sequencing? Plot what the pyro sequencing data would look like from the above DNA sequencing experiment **(5 points)**.



+1 measure light intensity
+3 peaks for proper bases
+1 correct axis labels

Name with Last Name, First: **Student ID:**

Question 4: Protein binding (25 points)

An integrin is a membrane protein that is responsible for cell adhesion by binding extracellular matrix. Suppose that we identify a new peptide sequence that integrins can bind using the phage display technique. The resulting peptide has a tripeptide consensus binding sequence: Arg-Gly-Glu (RGD).

a) Explain the experimental procedure of phage display up to isolation of the RGD peptide **(5 points)**.

   1. Construct combinatorial DNA library through error prone PCR, DNA shuffling, or combinatorial DNA synthesis
   2. Clone DNA into phage coat protein within phage vector
   3. Infect E. coli with phage to amplify
   4. Apply phage to substrate coated with immobilized integrins
   5. Wash non-specific phage
   6. Elute bound phage
   7. Infect E. coli with eluted phage to amplify
   8. Sequence the phage vectors to determine the peptide sequences displayed and determine a consensus sequence, in this case RGD
   9. Repeat if necessary

+2 create DNA library and clone into phage vector
+2 apply phage to immobilized integrins, wash non-specific, and elute bound
+1 amplify bound phage and sequence

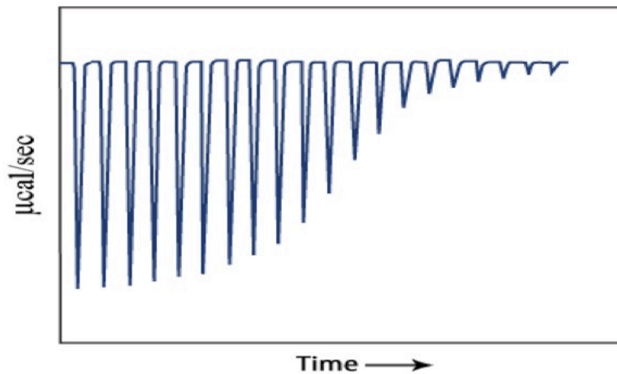
b) Suppose that we will synthesize the RGD tri-peptide using solid-phase peptide synthesis approach. Explain the steps required to accomplish this **(5 points)**.

   1. Start with bead functionalized with peptide linker
   2. Add Fmoc protected (N-terminus) glutamate, wash
   3. Remove Fmoc from glutamate with TFA, wash
   4. Add Fmoc protected glycine, wash
   5. Remove Fmoc from glycine with TFA, wash
   6. Add Fmoc protected arginine, wash
   7. Remove Fmoc from arginine with TFA, wash
   8. Cleave peptide from bead

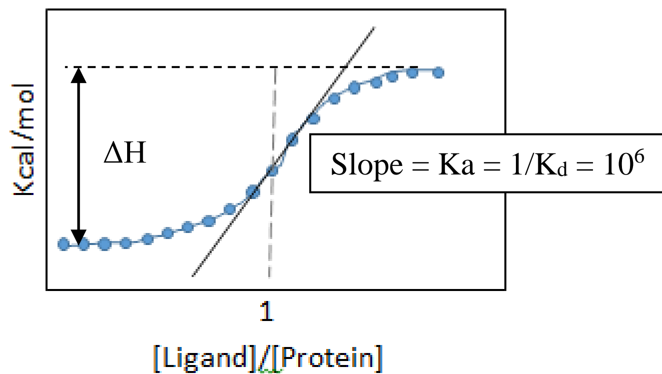
   +1 Start with functionalized bead
   +2 Add Fmoc protected amino acids
   +1 Add amino acids in correct order (C to N)
   +1 Deprotect after each amino acid addition

Name with Last Name, First: **Student ID:**

c) We found that the RGD peptide has $K_d = 10^{-6}$ M for integrins. We will characterize the binding dissociation thermodynamics between integrin and RGD tripeptide using isothermal calorimetry. Suppose that we prepare 1 mL of the integrin in 10µM concentration. Now, we prepare a 10 µM of the RGD peptide solution and make 20 drops of 100µL each after the equilibrium of the previous drop. Draw the expected isothermal calorimetry chart with heat exchange amount (µcal/s) on the y-axis and time (second) on the x-axis **(3 points)**. Convert the chart that obtained above to Kcal/mol of Ligand on y-axis and x-axis with Ligand/protein ratio **(3 points)**.



+1 One peak for each drop
+1 Correct shape of curve
+1 Correctly labelled axes



+2 Correct shape of curve
+1 Correctly labelled axes

d) Explain how you would obtain enthalpy change (ΔH) and K_d value from the above chart **(4 points)**

ΔH is the difference in kcal/mol between the point when all added ligand binds and when no added ligand binds as shown in the plot above. K_d is calculated as the inverse of the slope at the inflection point.

+2 ΔH, +2 K_d

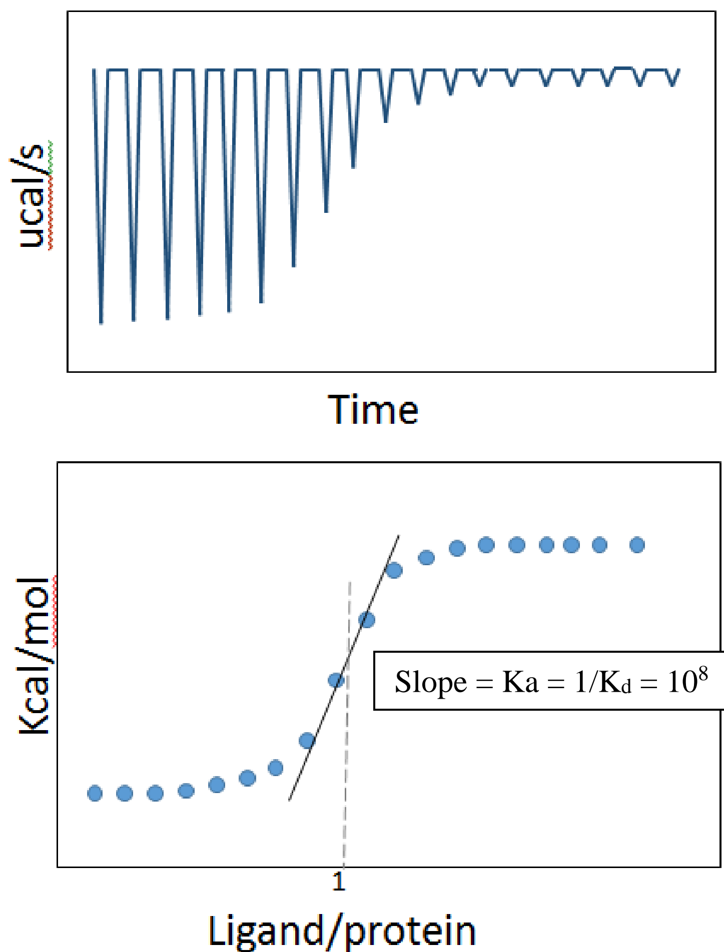
Name with Last Name, First: **Student ID:**

d) Cyclic RGD possesses a stronger binding affinity ($K_d=10^{-8}$ M) than the linear RGD peptide that we discussed above. Suppose that we synthesize the cyclic RGD peptide and perform the above experiment. How will the ITC results of the cyclic RGD change compared to linear RGD? **(5 points)**

The integrins will saturate with cRGD faster than with lRGD, and the slope at the inflection point of the kcal/mol vs. [L]/[P] plot will be much greater (10^8 instead of 10^6). [L]/[P] will still be equal to 1 at the inflection point. ΔH may or may not change.

+3 Increase in slope at inflection point
+1 Saturate faster
+1 Inflection point does not change

*New plots not necessary but will be used for grading if present



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Question 5: Group Projects (9 points)

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Imatinib (Gleevec),	Quetiapin (Seroquel),	Lactase,
Adalimumab (Humira),	Sildenafil (Viagra),	Trastuzumab (Herceptin),
Olanzapine (Zyprexa),	Memantine,	Rituximab (MabThera),
Bevacizumab (Avastin),	Etanercept (Enbrel),	Cyclosporine

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a. Which are antibody based therapeutics? **(3 points)**

Adalimumab
Bevacizumab
Trastuzumab
Rituximab

+0.75 each

b. Which are cancer therapeutics? **(3 points)**

Imatinib
Bevacizumab
Trastuzumab
Rituximab

+0.75 points each

c . Which medications can compete with neurotransmitters in their therapeutic action? **(3 points)**

Olanzapine
Quetiapin
Memantine

+1 each

Name with Last Name, First: **Student ID:**

Question 6: Protein therapeutic discovery and characterization (15 points)
Suppose that we discovered new class of tumor necrosis factor (TNF) called TNF-gamma which is released by macrophages. We decide to develop a new TNF-gamma interacting protein as a potential therapeutic for an immune disease using the yeast-2-hybrid system.

a) Describe how the yeast-2-hybrid system works **(5 points)**?

The yeast 2-hybrid system screens for protein-protein binding interactions within a yeast cell. A transcription factor required for the transcription of a report gene (often LacZ) is separated into its DNA binding domain and its activating domain. These two transcription factor domains are used to generate two fusion proteins. The protein fused to the DNA binding domain of the transcription factor is typically known and is called the bait. A library of unknown proteins or peptides are individually fused to the activating domain, which is called the prey. Binding of the fusion proteins brings the DNA binding and activating domains of the transcription factor in close enough proximity to enable transcription of the reporter gene, thus reporter gene transcription indicates a binding interaction between the two fusion proteins.

+1 separate DNA binding and activating domains of transcription factor
+2 generate fusion proteins
+2 reporter gene expression indicates binding

b) Suppose that we identify a good binding protein (protein-A) against TNF-gamma. We decide to characterize the protein-A binding affinity using surface plasmon resonance (SPR) spectroscopy. Design an SPR experiment and explain how we can determine the binding constant (K_d) using the kinetic measurements from the SPR **(10 points).**

1. Immobilize TNF-gamma onto the sensor surface
2. Flow protein A across the immobilized TNF-gamma
3. Record the change in reflectance as protein A binds TNF-gamma
4. When the TNF-gamma is saturated and the reflectance is no longer changing, begin flowing sample buffer lacking protein A
5. Record the change in reflectance as protein A dissociates
6. Repeat with 3-4 concentrations of protein A
7. Calculate kinetic constants for each concentration of protein A using derived mathematical kinetic descriptions of the association and dissociations phases
8. Using average kinetic constants from 7, calculate $K_d = k_d/k_a$

+2 Flow protein A over immobilized TNF-gamma
+2 At saturation, flow solution without protein A
+2 Repeat with multiple concentrations (span 0.1 to 10 times expected K_d with technical replicates)
+2 Use sensorgram data to calculate k_a and k_d using derived kinetic descriptions of association and dissociation phases
+2 $K_d = k_d/k_a$