1. (25 pts) Insulin is an important hormone produced by the pancreas that allows cells to process glucose. Diabetes mellitus, affecting 16 million people in the U.S., is a condition in which insulin production or utilization is impaired.  Current treatment for diabetes often involves subcutaneous injection of insulin.

a) Access the RCSB Protein Data Bank at http://www.pdb.org/pdb. Perform a search on insulin and access the human insulin two disulfide model to answer the following:

i) (2 pts) What is the primary chemical structure for insulin?

**A chain (21 residues): GIVEQSCTSISSLYQLENYCN**
**B chain (30 residues): FVNQHLCGSDLVEALYLVCGERGFFYTKPT**

ii) (3 pts) What is the secondary structure of insulin?

**The A chain exhibits an alpha helix formed by residues 13-16.   The B chain forms an alpha helix with residues 9-18.   The fractional alpha helical content for insulin is thus 14/51 = 0.27.**

**Disulfide bonds between cysteine residues A7-B7 and A20-B19 bond the A and B chains.**

iii) (2 pts) From the Ramachandran plot analysis, what is the range of psi, phi values for most of the amino acid residues in insulin?

**From the Ramanchandran plot, most of the residues are found in the range: $\phi = -45$ to $-120°$, $\psi = 0$ to $-80°$.**

b) (2 pts) Could insulin be readily radiolabeled with $^{125}$I for detecting its adsorption?

**$^{125}$I will react with tyrosine residues in proteins.  Insulin has 4 tyrosine residues and thus in principle can be radiolabeled with $^{125}$I.  In practice, the tyrosine at A14 appears to be the only one accessible for labeling (see P. Iozzo et al., Nucl. Med. Biol. 29, 73 (2002), and refs. therein).**

Numerous research efforts underway are investigating controlled release approaches that would eliminate the need for daily insulin injections.  Such strategies typically involve exposure of insulin to synthetic material surfaces.  To investigate the effects of adsorption on the secondary structure of insulin, Mollmann et al. (*Eur. J. Pharm. Sci.* **2006**, *27*, 194) performed circular dichroism (CD) measurements on insulin in phosphate buffered saline (PBS) solution and adsorbed to the surface of colloidal Teflon particles 215 nm in diameter and stabilized in water with a small fraction of negatively charged sulfate groups on their surface.  The CD data are shown in the figure below.

c) (3 pts) What is the effect of adsorption on the secondary structure of insulin?

Image removed for copyright reasons.

See Fig. 2 in Mollmann, Susanne H., Lene Jorgensen, Jens T. Bukrinsky, Ulla Elofsson, Willem Norde, and Sven Frokjaer.  "Interfacial Adsorption of Insulin: Conformational Changes and Reversibility of Adsorption." *Eur J Pharm Sci* 27 (2006): 194-204

**The alpha helical content of insulin decreases upon adsorption while the random coil content increases.  This is observed through the decreased negative ellipticity at 222 nm and decreased positive ellipticity at 192 nm (Note that a random coil exhibits a large negative ellipticity at 197 nm.)**

d) (4 pts) From the CD data provided, calculate the fractional $\alpha$ helical content for insulin in solution and adsorbed to the Teflon particles.

**The helical content can be determined using the equation:** $f_{\alpha-helix} = \dfrac{-[\theta]_{222}}{40,000}$

**The molar ellipticity at 222 nm is ~ -12,000 for insulin in solution, giving $f_\alpha = 0.30$, in good agreement with 0.27 determined above from the structure database.  Adsorbed to a surface, the $[\theta]_{222}$ ~ -9000, giving $f_\alpha = 0.23$.**

e) (2 pts) Why might changes in the secondary structure of insulin be important to consider in developing controlled delivery strategies?

**If insulin denatures upon contacting a synthetic surface before being administered, its activity in vivo may be reduced.**

Mollmann et al. further studied the adsorption of FITC-labeled insulin to determine if the fluorescent dye changed the adsorption kinetics.  For this experiment, mixtures of FITC-labeled and unlabeled insulin were prepared in different ratios to reach the same total insulin concentration (a concentration that corresponded to near monolayer coverage). They found that the fluorescence intensity increased linearly with the fraction of FITC-labeled insulin in solution, and concluded from this result that FITC-labeled and unlabeled insulin have comparable affinities for the Teflon beads.

f) (7 pts) Do you agree with their conclusion?  Develop a model to describe the fractional coverage ($v_F$) for the FITC-labeled component of the mixed insulin solution, based on a modified Langmuir model.  Under what conditions does $v_F$ scale linearly with the fraction of FITC-labeled insulin [$P_F$]?

**Building on the Langmuir model, in dynamic equilibrium we should expect both FITC-labeled and unlabeled insulin molecules to adsorb and desorb from the surface at the same rate:**

$$k_{a,F}[P_F][S] = k_{d,F}[P_F S]$$
$$k_{a,U}[P_U][S] = k_{d,U}[P_U S]$$
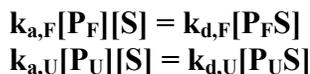
**where U= unlabeled and F = FITC-labeled.  The corresponding affinity constants will be:**

$$K_F = \frac{[P_F S]}{[P_F][S]} \qquad K_U = \frac{[P_U S]}{[P_U][S]}$$

**From here we can define the fraction of sites filled by FITC-labeled insulin as:**

$$v_F = \frac{[P_F S]}{([S]+[P_U S]+[P_F S])} = \frac{K_F[P_F]}{(1+K_U[P_U]+K_F[P_F])}$$

**Noting that for these experiments, the total polymer concentration is a constant:**

**[P] = const = [$P_U$] + [$P_F$],**

**we can substitute for [$P_U$]:**

$$v_F = \frac{K_F[P_F]}{1+K_U[P]+(K_F-K_U)[P_F]} = \frac{K_F[P_F]}{C+(K_F-K_U)[P_F]}$$

**where $C$ is a constant.  From this result we find that if $K_F = K_U$, then $v_F$ should vary linearly with [$P_F$].**

**One must be careful in performing the experiment, however.  At high [P], if $K_U$ is large compared to $K_F$ so that $C$ is large compared to $(K_F - K_U)[P_F]$ (small values of [$P_F$]), then:**

$$v_F \approx \frac{K_F[P_F]}{C}$$

**so that a linear increase in fluorescence intensity with [$P_F$] might still be observed. The authors provided insufficient details on their experiments to determine whether their conclusion is valid or not.**

2.  (20 pts) Prosthetic vascular grafts are commonly employed in the treatment of atherosclerosis, as a means to bypass occluded blood vessels.  Read through the text on pp. 479-483 (1$^{st}$ ed.: pp. 420-422) to assist in answering the questions below.

a) (2 pts) What materials are currently used in commercial vascular grafts?

**Dacron (polyethylene terephthalate, PET) is commonly used for large diameter vessels while expanded polytetrafluoroethylene (ePTFE) is used for synthetic grafts of diameter below < 8 mm.**

b) (2 pts) What is the most common reason for failure of synthetic vascular grafts of small diameter?

**Thrombosis is the chief source of failure in small diameter grafts, as even moderate deposits on the inner surface of the graft can restrict blood flow.**

c) (4 pts) Describe the expected synthetic/biological material interface at the interior and exterior surfaces of a vascular graft many months post-implantation.

**The interior of the graft will typically have an inner lining of aggregated platelets and fibrin, except near the sutured ends of the graft where endothelial cells migrate into the device to create a neointima.  The exterior of the graft will exhibit a histology that is typical of the foreign body response, namely a fibrous capsule encompassing the device, incorporating foreign body giant cells adjacent to the device surrounded by connective tissue containing fibroblasts and capillaries.**

Fibrinogen is known to play an important role in clot formation.  Makogonenko et al. (*Biochemistry* **2002**, *41*, 7907) investigated the interaction of adsorbed fibrinogen with fibronectin, employing surface plasmon resonance (SPR).   The figure below shows fibronectin adsorption data at 22 °C on a gold surface coated with fibrinogen (dashed line, 100 nM fibronectin), and the same surface after subsequently being treated with thrombin (solid lines, 0.25 – 3.0 nM fibronectin).

Image removed for copyright reasons.

See Fig. 2(a) in Makogonenko, E., G. Tsurupa, K. Ingham, and L. Medved. "Interaction of Fibrin(ogen) with Fibronectin: Further Characterization and Localization of the Fibronectin-Binding Site." *Biochemistry* 41 (2002):  7907-7913.

d) (2 pts) What do the units of SPR response indicate?

**The response is measured in arc seconds, the shift in the angle where maximum energy transfer occurs to the Au film.  This value increases with fibronectin adsorption because the index of refraction at the surface is increased.**

e) (2 pts)  Explain why fibrinogen binds fibronectin only after exposure to thrombin.

**Thrombin catalyses the cleavage of fibrinogen to form fibrin.  The results suggest that the fibronectin binding sites on fibrinogen are only accessible when fibrinogen is cleaved.**

f) (2 pts) From the inset of $k_{obs}$ data obtained from the fits to the SPR data for thrombin-exposed fibrinogen, estimate the value of the dissociation constant $K_D$.

**Noting $k_{obs} = k_d + k_a [P]$, $k_a$ should be given by the slope and $k_d$ by the y-intercept of the straight line fit.**

$$K_D = k_d / k_a \approx 0.3 \ nM$$

g) (3 pts) Based on the $K_D$ value you obtained for (f), would you expect fibronectin to bind to adsorbed fibrinogen *in vivo*?  Explain your answer, stating any assumptions you make.

**The $K_D$ value provides a metric for the solution concentration required for substantial adsorption to occur to a given surface (at [P] = $K_D$, half the surface sites are occupied).  The concentration of fibronectin in plasma is ~0.3 mg/mL, or 1250 nM (molecular weight of fibronectin is 240,000 g/mol), which is well above the observed $K_D$, so fibronectin adsorption might be expected.   It should be noted, however, that this analysis does not account for other proteins in the blood stream, which might adsorb preferentially over fibronectin.**

h) (3 pts) What role does adsorbed fibronectin have in the body's response to an implanted biomaterial?

**Fibronectin is an adhesion protein that contains peptide sequences that bind to integrins on the surface of platelets.  When adsorbed on a surface, fibronectin allows platelets to adhere to the surface to initiate clot formation.  Adsorbed fibronectin also binds integrins on fibroblasts, enabling their recruitment to the site of injury to initiate the formation of new connective tissue.**