1. (38 pts) Amyotrophic lateral sclerosis, ALS, also known as Lou Gherig's disease, is a neuromuscular disorder in which patients gradually lose peripheral motor activity. One approach under investigation for treatment of ALS combines gene therapy with cell encapsulation therapy. In the ALS treatment under consideration, rodent cell lines are modified genetically to release ciliary derived neurotrophic factor, CNTF, an agent which has shown promise for slowing the deterioration of neurons in more conventional preclinical drug tests. In conventional cell culture studies, Hamster-BHK cells transfected with the gene for CNTF, showed promisingly high secretion rates of 1 ng/10^6cells/day.
You are working for a company that is developing a cell encapsulation device to house the Hamster-BHK cells in vivo. Prototype membranes having the porosity characteristics given in the table below have been developed.

(a) What functions does the membrane serve in this device? (3 pts)

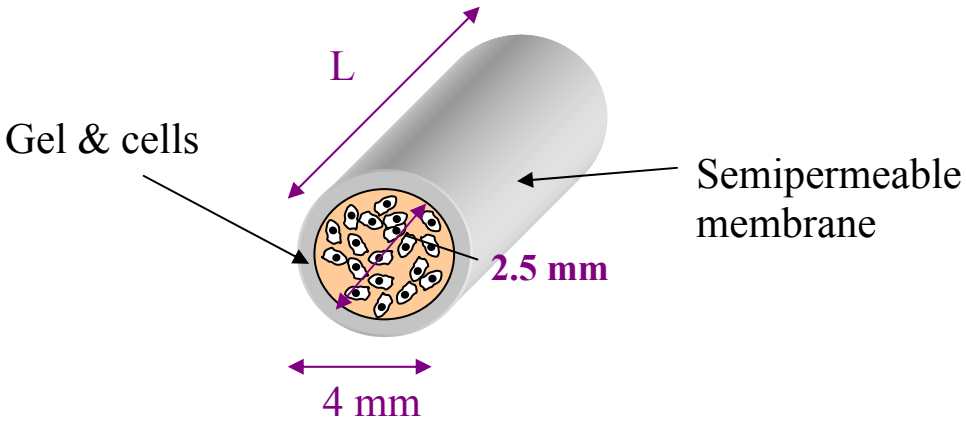
The membrane serves to contain the cells as well as isolate them from the immune system while still allowing for permeability of nutrients, waste and CNTF.

(b) What processing route would be used to make the membrane for this device? What would be a typical material for such a membrane? (4 pts)

A membrane could be formed using immersion precipitation of PAN-PVC, PP, polycarbonate, cellulose nitrate, or acrylic polymers. In this process, a polymer solution is coated onto a cylindrical substrate and the substrate is dipped into a coagulation bath that contains a poor solvent for the polymer. The polymer precipitates onto the substrate and subsequent removal of the substrate leaves a hollow fiber membrane.

(c) Which prototype membrane will exhibit the highest CNTF delivery rates, assuming the same number of cells is contained in each device? Justify your response quantitatively, stating any assumptions you make. (6 pts)

Membrane Prototype	1	2	3
Pore size (Å)	16±7	27±7	46±20
Surface Porosity (%)	0.044±0.008	0.045±0.006	0.053±0.006
Thickness (μm)	670±25	810±14	819±22



By substituting $D_{eff} = \frac{D_{pore}\varepsilon}{\tau}$

into $\frac{dM_t}{dt} = \frac{DKA}{\delta}(C_2 - C_1)$

the result is:

$$\frac{dM_t}{dt} = \frac{D_{pore}KA\varepsilon}{\delta\tau}(C_2 - C_1)$$

Assuming that the internal and external concentrations within the different systems do not change, that the tortuosities of each membrane are equal, and that the devices are of the same size and have the same distribution coefficient K, $\frac{dM_t}{dt}$ **only depends on** $\frac{\varepsilon}{\delta}$

Membrane Prototype	1	2	3
Surface Porosity ε (%)	0.044	0.045	0.053
Thickness δ (μm)	670	810	819
ε/δ	6.56716E-05	5.56E-05	6.47E-05

Membrane prototype 1 has the highest ratio of surface porosity to thickness and should exhibit the highest CNTF delivery rate.

(d) Approximately how long should this device be (L) to hold 10^6 cells? State any assumptions you make in arriving at your value. (4 pts)

An approximate cell diameter is 10μm, and approximating the cell as a sphere to calculate the cellular volume yields 5.24E-7mm^3/cell. This volume times 10^6 cells yields 0.524mm^3. Solving for the length of an inner chamber of this volume and a radius of 1.25mm results in a length of 0.11mm. This does not account for extracellular space, and the device length would have to be increased by the percent of unfilled volume within the device to accommodate a realistic cell density.

$$\frac{4}{3}\pi(5x10^{-3}\,\text{mm})^3 = 5.24\text{E-}7\,\frac{\text{mm}^3}{\text{cell}}$$

$$\frac{5.24\text{E-}7\text{mm}^3}{\text{cell}}(10^6\text{cells}) = 0.524\text{mm}^3$$

$$0.524\text{mm}^3 = \pi(1.25)^2 l \quad l = 0.11\text{mm}$$

(e) Based on pore size considerations, would any of the prototype membranes be expected to exclude the antibody IgG (molecular weight 160,000 g/mol)? Justify your response as quantitatively as possible, stating any assumptions you make. (5 pts)

An approximate protein density is 1.4g/cm^3, and a protein of this MW would have a linear dimension of about 57x10^{-8} cm. The membrane with the largest pore size has a pore linear dimension of (46±20)x10^{-8} cm and therefore is not small enough to exclude IgG.

$$\frac{160{,}000g}{mol}\,x\,\frac{mol}{6.02x10^{-23}\,proteins}\,x\,\frac{cm^3}{1.4g} = \frac{1.90x10^{-19}cm^3}{protein} \qquad \left(1.90x10^{-19}cm^3\right)^{\frac{1}{3}} = 57x10^{-8}cm$$

linear dimension of largest pore = 4.6+2.0nm>5.7nm = linear dimesion of protein

(f) In prototype devices prepared by your company, cells were seeded in a polyethylene oxide (PEO) hydrogel within the membrane, and cultured in nutrient-rich media to test for CNTF release. To your management's dismay, the release rate was found to be much lower than anticipated from studies of the same cells seeded on conventional culture plates. Moreover, CNTF secretion was seen to decrease monotonically over several days in culture. Provide <u>two</u> likely explanations for these observations. (4 pts)

The device diameter is too large for nutrients to reach cells in the center of the device (the tube edges would be sealed). An approximate limit to the distance cells can be separated from a nutrient source is 0.5mm, so the inner diameter should be no larger than 1mm, but in this device it is 2.5mm. In addition, the PEO hydrogel does not contain any binding epitopes for the cells, and does not support protein adsorption needed for cell attachment and proper functioning.

(g) Suggest design changes to the device to rectify the problems noted in (f). (4 pts)

To provide binding epitopes for cells, the membrane could encapsulate cells seeded into a network of collagen. To remedy transport problems, the device dimensions could be decreased or the

geometry of the device could be changed to a disc. If a tubular device with an inner diameter of 1mm were fabricated, it would have to be 0.67mm long to encapsulate 10^6 cells.

(h) Would you expect this device to be more resistant to crushing in the longitudinal or transverse direction? Justify your answer quantitatively. (5 pts)

This device would be more resistant to crushing in the longitudinal direction where the modulus of the device is related to the moduli of the two materials by the following equation:

$$E_{composite} = V_1 E_1 + V_2 E_2$$

wheras the modulus of the device in the transverse direction would follow:

$$\frac{1}{E_{composite}} = \frac{V_1}{E_1} + \frac{V_2}{E_2}$$

The resulting modulus of the device in the transverse direction is less than the modulus of the device in the longitudinal direction for all reasonable values of E_1 and E_2
For example, the volume fraction of the membrane is about 0.6 and that of the PEO gel is about 0.4, as estimated using the device dimensions. Assuming a compression modulus of 2GPa for the membrane and 0.2GPa for the hydrogel, the modulus of the device in the transverse direction is 0.434GPa and the modulus in the longitudinal direction is 1.28GPa.

(i) Your company is also considering development of cell encapsulation therapies for type I diabetes based on these membranes. Would you recommend pursuing this application? Explain your answer. (3 pts)

This device geometry would not be practical for diabetes treatment. Approximately 10^9 cells would be needed to produce a therapeutic level of insulin, and using the same reasoning as above, a device of this nature would need to be 11 cm long to encapsulate 10^9 cells.

2. (34 pts) Expanded polytetrafluoroethylene (ePTFE) is a commonly used material for synthetic vascular grafts. One mode of failure of such devices is via bacterial infection initiated during implantation. Upon the introduction of a foreign material into the body, a competition for the surface ensues between bacteria and leukocytes that serve to fight infection. One hypothesis for elevated infection rates for PTFE graft implantations is that leukocyte migration is impeded on ePTFE surfaces, allowing bacteria to become established in the early stages following implantation.

(a) Describe the process by which leukocytes are attracted and migrate to the site of a vascular graft implantation. (4 pts)

Complement activation by the alternative pathway occurs when activation of endothelial cells at the site of injury results in cleavage of C3 protein and the deposition of C3b on the graft surface. The C3b fragment catalyzes the cleavage of C5 to C5a, which is a soluble ligand for specific receptors expressed on the leukocyte cell surface. This recognition of C5a allows for chemotactic attraction and migration of leukocytes to the site of implantation.

To investigate which integrin subunits play a significant role in leukocyte migration on ePTFE, Chang and coworkers performed random migration studies on populations of fluorescently labeled polymorphonuclear leukocytes (PMN's or neutrophils) exposed to antibodies for different integrin subunits. Confocal microscopy images depicting PMN migration on ePTFE after 3 h of incubation with IgG (A) or anti-CD18 (C) are shown below.

(b) Explain how one would obtain a motility coefficient for the PMN's from such data. (4 pts)

For random migration, the ratio of PMN concentration at a distance x from the cell well after a certain time t (3 hours in this case) to the concentration of PMN in the well is described by the following equation:

$$\frac{c(x,t)}{c_0} = efrc\left(\frac{x}{4\mu t}\right)$$

A fit to a plot of the PMN concentration ratio versus distance obtained from this data could be used to find μ, the motility coefficient.

(c) Provide a molecular level explanation for the observed differences in migration between PMN's exposed to anti-CD18 vs. those exposed to IgG. (3 pts)

When bound to anti-CD18, the CD-18 integrin subunit is unable to participate in binding with the substrate, thereby decreasing cellular adhesion to the substrate and subsequent migration.

Images removed for copyright reasons.

See Fig. 4(a) and (c) in Chang, Charlie C., Rene S. Rosenson-Schloss, Tanuja D. Bhoj, and Prabhas V. Moghe. "Leukocyte Chemosensory Migration on Vascular Prosthetic Biomaterial is Mediated by an Integrin ß2 Receptor Chain." *Biomaterials* 21 (2000): 2305-2313.

As a potential surface modification method for ePTFE, Kidd and Williams investigated temporarily seeding epithelial cells onto ePTFE to deposit extracellular matrix proteins and thus potentially improve endothelial cell adhesion and vascularization of new tissues. In their study, 6 different cell types were seeded on ePTFE: human microvessel endothelial cells (HMVEC), rat microvessel endothelial cells (RMVEC), human squamous epithelial cell line (HaCaT), a tumorigenic variant of HaCaT (II-4), rat bladder squamous cell carcinoma cell line (804-G), and lung carcinomatous epithelial cell line (A549). Cells were seeded at equivalent densities and left in culture for 8 days, after which they were removed using ammonium hydroxide, which allowed deposited ECM proteins to remain on the surface. Subsequently, ECM was collected from the surface of the ePTFE with a rubber scraper and SDS polyacrylamide gel electrophoresis was performed to determine the proteins present. Western blot analysis was performed employing rabbit or mouse antibodies for collagen I, collagen IV, fibronectin, laminin-1 and laminin-5 in different lanes of the gel, followed by a secondary antibody, rat antimouse IgG or goat antirabbit IgG, conjugated to horseradish peroxidase. Results from the SDS-PAGE study are shown below.

(d) Explain the function of the two antibodies used in the Western blot analysis. (4 pts)

The first adhesion protein-specific rabbit or mouse antibodies bind to their respective adhesion protein. The second antimouse and antirabbit antibodies then bind to the first, protein specific antibodies. These anti-antibodies are linked to an enzyme (horseradish peroxidase) that catalyzes formation of a colored by-product when in the presence of a chromatogenic agent, allowing for detection of the (enzyme-antibody)-(antibody)-(adhesion protein) complex.

(e) Based on the SDS-PAGE results, which protein has a higher molecular weight, fibronectin or laminin-5? Explain. (2 pts)

Images removed for copyright reasons.

See Fig. 1 in Kidd, Kameha R., and Stuart K. Williams. "Laminin-5-enriched Extracellular Matrix Accelerates Angiogenesis and Neovascularization in Association with ePTFE." *J Biomed Mater Res* 69A (2004): 294-304.

SDS binds (~1 SDS per 2 amino acids) and denatures proteins, at which point the native charge becomes negligible and the charge/mass of each protein is essentially constant. The bound proteins are then placed under an electric field and forced to move through a gel. This separates the protein chains via molecular weight as the low molecular weight species move through the gel media faster than the bulkier, higher molecular weight species. The results show that fibronectin is of a higher molecular weight because it does not travel as far as the laminin 5.

To ascertain the effects of ECM-modification on vascularization near the ePTFE grafts, discs of ePTFE modified as described above were implanted into the fatty tissue of rats. After 5 weeks, the devices were explanted and tissues in the vicinity of the implant examined for new vessel growth. Data quantifying angiogenesis and neovascularization are given in the figure below, reported as means and standard deviations of n=4 samples for RMVEC-, HMVEC, A549-, and II-4-modified samples and n=8 samples for 804-G, HaCaT and nonmodified samples.

(f) Describe the expected tissue morphology in the vicinity of the ePTFE implant after (i) five days; (ii) five weeks. (6 pts)

Within 5 days, monocytes recruited by C5a have evolved into macrophages, and may have fused together to form foreign body giant cells. Fibroblasts recruited by macrophage/FBGC products have started to deposit collagen, leading to the formation of pink granulation tissue, accompanied by capillary sprouting or angiogenesis. Within five weeks, significant connective tissue remodeling should have occurred, leading to the formation of a thin, encapsulating fibrous layer around the implant.

(g) Which of the ECM-modified samples exhibit a statistically significant increase in angiogenesis compared with the unmodified ePTFE control? (6 pts)

$$t = \frac{\langle x \rangle - \langle x' \rangle - (\mu - \mu')}{\sigma_p \sqrt{\frac{1}{N} + \frac{1}{N'}}} \qquad \sigma_p = \sqrt{\frac{vS^2 + v'S'^2}{v + v'}}$$

Assume $\mu=\mu'$, if the t value falls between this interval:

$$-t_{\frac{1+P}{2}} < t < t_{\frac{1+P}{2}}$$

then the difference between the two samples is not statistically significant

for v=14, at 95%, $t_{.975}$ = 2.14 for comparison of 804-G and HaCaT (N=8) with control (N=8)

for v=10, at 95%, $t_{.975}$ = 2.23 for comparison of II-4 (N=4) with control (N=8)

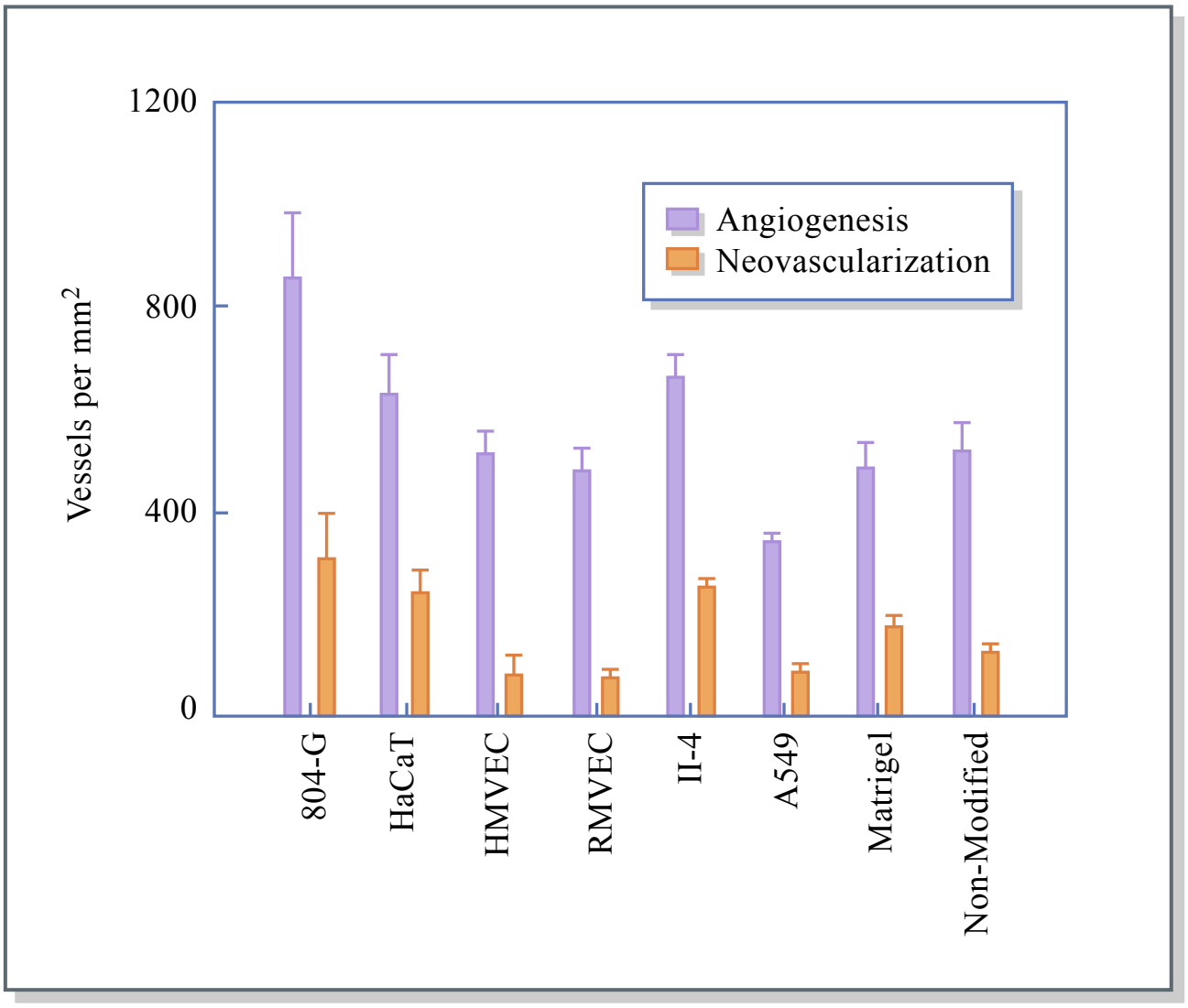
Sample	x	S	N	ν	σp	tcalc	t.975	Stat. Significant	
non-modified	510	70	8	7					
804-G	850	130	8	7	104.4031	6.513219	2.14	Yes	
HaCaT	640	70	8	7	70	3.714286	2.14	Yes	
II-4	660	50	4	3	64.65292	3.788676	2.23	Yes	

(h) From the data provided, give a possible explanation for your findings in (g). (2 pts)

Only the samples that contain laminin 5 result in a statistically significant increase in angiogenesis, suggesting that this protein plays an important role in the process.

(i) How might the results of this study be relevant to cancer therapies? (3 pts)

Angiogenesis is necessary for tumor growth to occur, and by interfering with laminin 5-cell interactions at the tumor site, angiogenesis and therefore tumor growth may be reduced. Particularly, the observed increase in angiogenesis with laminin 5 deposition occurred in epithelial cancer cell lines.



3. (39 pts) Over 15M people in the United States alone are afflicted with type I diabetes, a disease in which the pancreas fails to generate adequate insulin necessary for metabolizing glucose. The management of blood glucose is currently achieved by active monitoring of blood glucose concentration, typically ranging from 1-30 mM, along with daily insulin injections to replace or supplement the patient's natural hormone production.

(a) Describe the operation of commercial biosensor devices used for glucose monitoring. (4 pts)

Electrochemical detection of glucose is achieved in commercial biosensors through the immobilization of glucose oxidase near an electrode. The enzyme catalyzes the following reaction:

$$\text{Glucose} + O_2 + H_2O \xrightarrow[\text{GOD}]{} \text{Gluconic acid} + H_2O_2$$

The H_2O_2 produced by this reaction is subsequently oxidized at the electrode, creating a measurable current:

$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$

As a potential new method for glucose monitoring, Hsieh et al. developed a surface plasmon resonance device. For this device, bacterially-derived glucose/galactose-binding protein (GGBP), a known receptor for glucose, was covalently attached to the SPR surface by replacing specific amino acids in the structure by cysteine (R group= CH_2SH) through genetic mutation. Below is a ribbon diagram of GGBP showing its binding pocket for glucose and locations of mutation sites.

(b) Sketch the general reaction employed to bind the GGBP to the SPR surface. (2 pts)

(c) Is this device a biosensor? What constitutes the detection element, analyte and transducer? (4 pts)

This device uses a biological agent for detection of the analyte and can therefore be classified as a biosensor. The GGBP serves as the detection element, the analyte is glucose, and the measurable shift in SPR signal upon glucose-GGBP binding serves as the signal transducer.

Image removed for copyright reasons.

See Fig. 1 in Hsieh, H. V., Z. A. Pfeiffer, T. J. Amiss, D. B. Sherman, J. B. Pitner. "Direct Detection of Glucose by Surface Plasmon Resonance with Bacterial Glucose/galactose-binding Protein." *Biosensors and Bioelectronics* 19 (2004): 653-660.

The figure below shows the SPR signal (RU= response units, 1RU ~1 pg/mm^2) generated upon the injection of 100 μM glucose in a running buffer solution flowing at 5 μl/min over a surface coated with GGBP mutated at the E149 site (solid line), and a second surface prepared with E149-mutated GGBP subsequently denatured by overnight exposure to 1M guanidine HCl (dashed line).

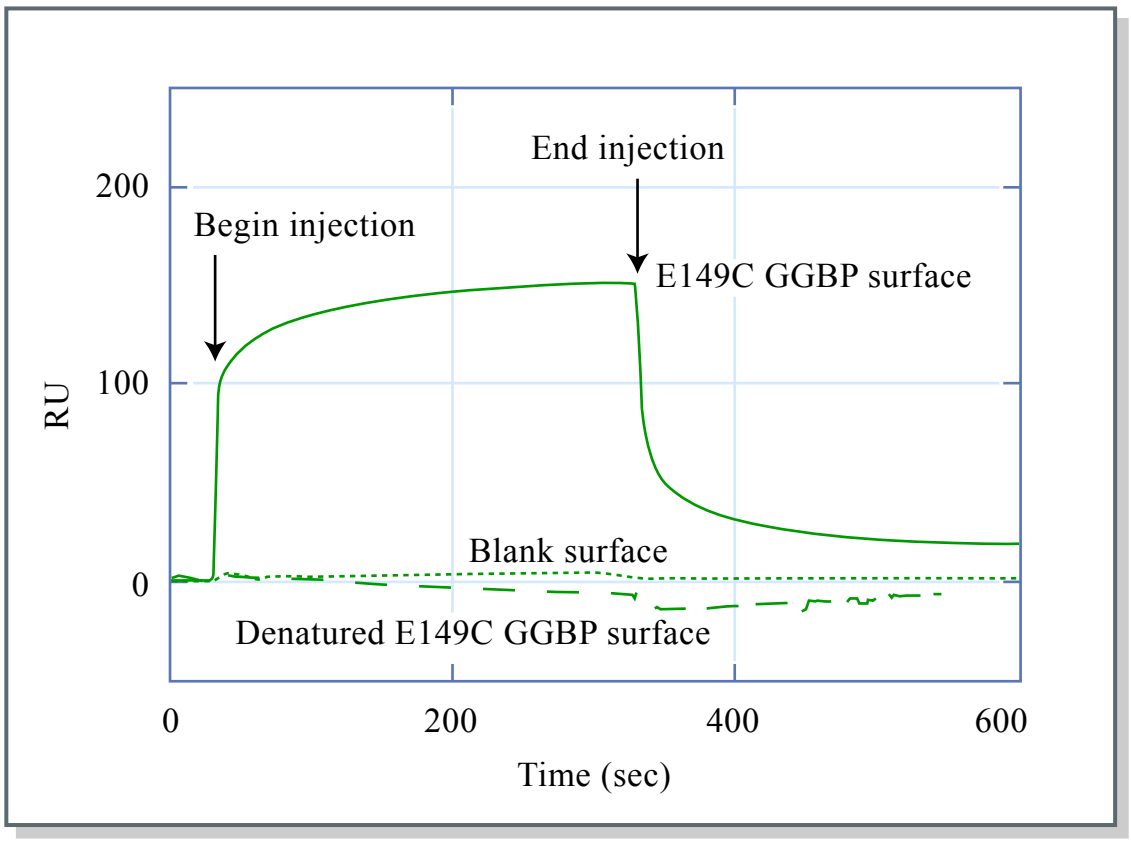


Figure by MIT OCW.

(d) Explain the observed features in the SPR signal as a function of time for the E149C mutant GGBP surface (solid line). (3 pts)

Upon the injection of glucose, binding occurs between glucose and the surface bound GGBP mutant, and this binding elicits a change in the SPR signal by causing a change in the refractive index of the surface, $n_{surface}$.

$$K_{sp} = \frac{\omega_0}{c}\sqrt{\frac{\varepsilon_{metal} n_{surface}^2}{\varepsilon_{metal} + n_{surface}^2}}$$

Once the injection stops, the SPR signal begins to return to its original value as glucose detaches from GGBP and is carried away by the running buffered solution. A fraction of glucose appears to be bound irreversibly, since the signal never returns to zero.

(e) Why does the SPR signal differ when E149C GGBP is denatured prior to glucose exposure? (2 pts)

An exact protein structure is essential to the formation of the glucose specific binding pocket, and upon denaturation, this exact structure is lost and the glucose specific binding region no longer exists in a functional form.

(f) What techniques could you use to characterize changes in the: (i) secondary structure content, (ii) surface chemistry, (iii) surface morphology and (iv) thickness of the GGBP layer after exposure to guanidine HCl? (8 pts)

Each technique must be capable of sample analysis in an aqueous environment:
Circular Dichroism could be used to detect the secondary structure content by making colloidal gold spheres modified with GGBP.
Surface chemistry could be analyzed with an advancing/receding contact angle.
AFM could be used to evaluate surface morphology by looking at height information in tapping mode.
The thickness of the GGBP layer could be calculated with in-situ ellipsometry.

In a series of experiments, the SPR signal change was recorded as a function of glucose concentration for a E149C mutant GGBP surface and a GGBP with cysteine mutations at E149C, A213S and L238S, as shown below.

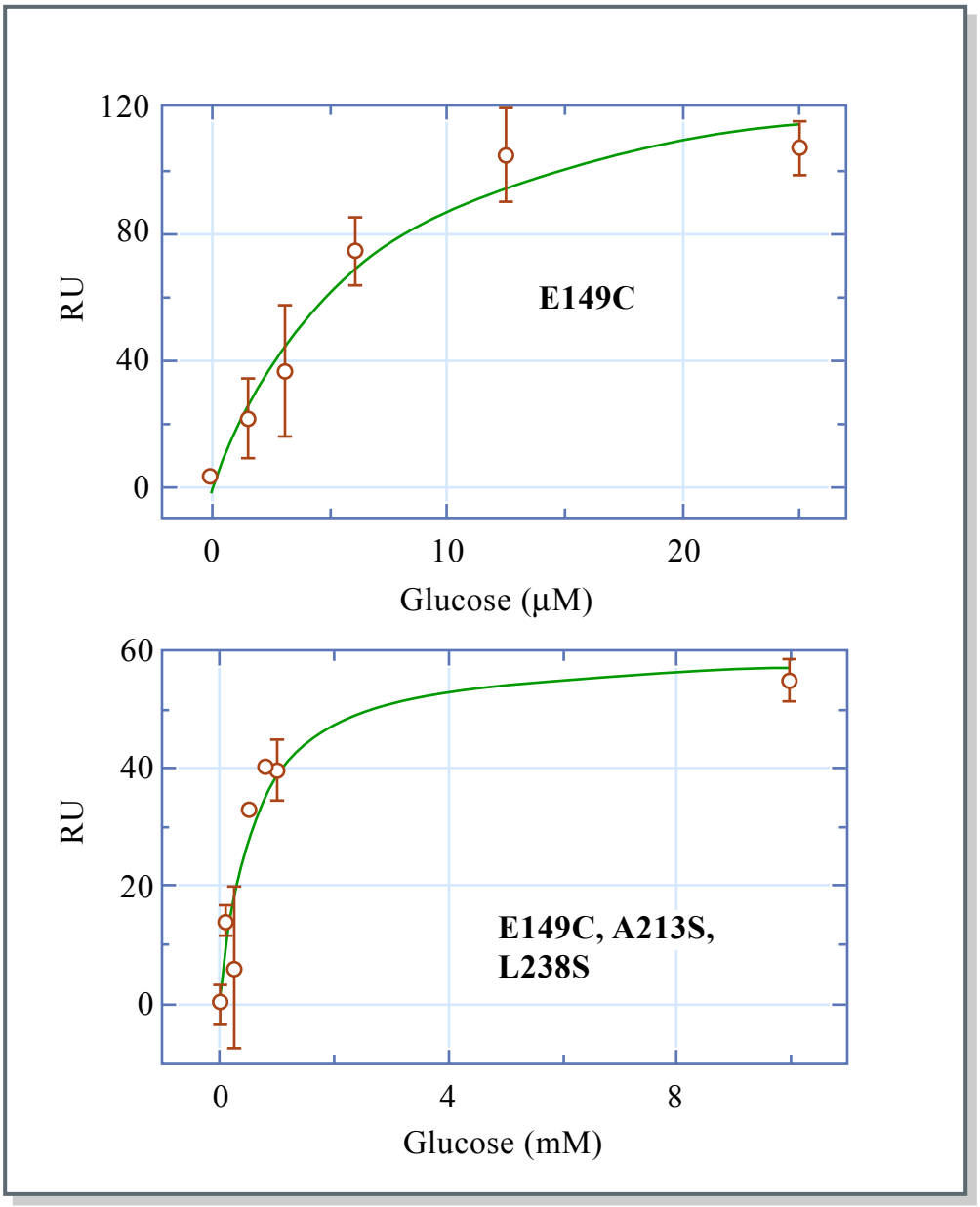


Figure by MIT OCW.

(g) From the data provided, calculate the equilibrium dissociation constant for glucose-GGBP binding in each case. (6 pts)

Noting that $\nu = RU/RU_{max}$, the Langmuir equation can be expressed as:

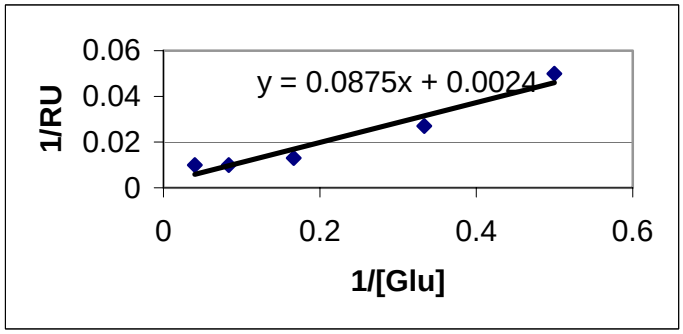
$$\frac{1}{RU} = \frac{K_d}{[Glu]RU_{\max}} + \frac{1}{RU_{\max}}$$

Performing linear regression on the data gives a best-fit line of:

1/RU = 0.088/[Glu] + 0.0024 from which we obtain RU_{max} = 425 and K_d = 37 uM

for E149C:

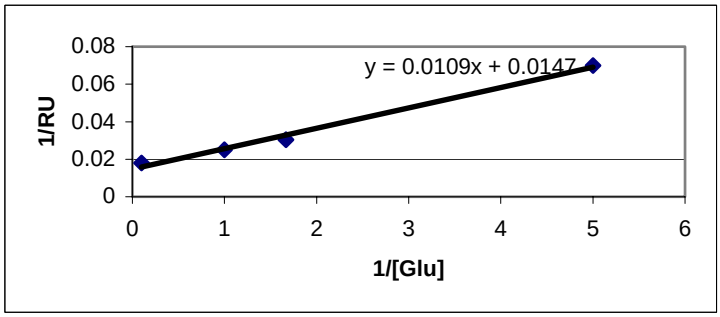
RU	[Glu] uM	1/RU	1/[Glu]
20	2	0.05	0.5
37	3	0.027	0.333333
75	6	0.013	0.166667
105	12	0.01	0.083333
105	25	0.01	0.04



1/RU = 0.011/[Glu] + 0.015 from which we obtain RU_{max} = 67 and K_d = 0.73 mM

for E149C, A213S, L238S:

RU	[Glu] mM	1/RU	1/[Glu]
14	0.2	0.07	5
33	0.6	0.0303	1.666667
40	1	0.025	1
55	10	0.018	0.1



(h) Explain the observed differences in K_d for the two surfaces. (3 pts)

By increasing the number of binding sites between the surface and GGBP, access of glucose to the binding pocket is decreased, making it harder for glucose to bind, resulting in an increase of the dissociation constant.

(i) How would you expect the value of K_d to change if the cysteine mutation was performed at the K137 site? (2 pts)

By attaching GGBP via the K137 site, the ability of glucose to access the binding pocket should increase, making it easier for glucose to bind, resulting in a decrease of the dissociation constant.

(j) Would either of the sensors above be useful for monitoring glucose levels typical in humans? Explain. (3 pts)

Blood glucose levels in humans are typically close to mM concentrations, and therefore the sensor attached by multiple binding sites may be useful for monitoring glucose levels in humans.

(k) Provide one key drawback to the proposed SPR sensor for blood glucose monitoring. (2 pts)

Non-specific adsorption of other blood components could occur and result in a SPR signal change as well. A membrane that limited surface access to small molecules like glucose would be useful in this case.