Analyzing the Function of Cartilage Replacements

Purdue University
EDGE Summer Camp

Summary
This laboratory module introduces basic principles and applications of chemical engineering as applied to the fields of biomaterials and tissue engineering. You will learn about the history of and need for tissue replacements. Specifically, we will discuss replacements for diseased or damaged cartilage and some of the goals and challenges that remain. You will participate in a hands-on laboratory that uses one of the techniques for evaluating the quality of cartilage replacements.
Engineering Disciplines: Chemical Engineering, Biomedical Engineering, Materials Science and Engineering, Mechanical Engineering

The project involves determining the concentration of a common cartilage component. You will learn one of the methods that many engineers use to evaluate the success of their potential cartilage substitutes. You will calculate the concentrations of six prepared samples and then perform a color-based chemical reaction on these samples. You will use the measurements of the prepared samples to determine the concentrations of two samples of unknown concentrations. As is typical in an engineering environment, you will work in teams, run experiments to collect information, evaluate and analyze your data, make some calculations and graphs, and draw conclusions from your results.

Background
Articular Cartilage

- Function: Covers and cushions joints, which prevents bones from rubbing together
- Motivation for Studying:
  - Joint injury increases chance of developing arthritis later in life
  - Osteoarthritis – degradation of articular cartilage
    - Estimated by 2030, 1 out of 4 Americans will have arthritis
  - Cartilage has limited capacity to self-heal
- Natural Structure – Biological Components and Function
  - Cells (chondrocytes) - secrete extracellular matrix (ECM)
  - Collagen - gives cartilage strength when pulled
  - Proteoglycans - gives cartilage strength when compressed

Characterizing and Measuring Cartilage Components (How do we know it is cartilage?)

One way to characterize cartilage is to take your sample and cut it into very thin sections. Dyes can be used to stain specific components, and you can examine these stained sections under a microscope. Another way to examine cartilage is to take your cartilage sample and break it up through enzymatic digestion. Once the sample is broken up, we can ask the question of how MUCH extracellular matrix components were produced by the cells.

To measure the amount of glycosaminoglycan (GAG), a proteoglycan, we will use a colorimetric reaction:

Acidic GAG + Basic Dye $\longrightarrow$

(Acidic GAG)(Basic Dye) Complex + water

To quantify the how much GAG was available for reaction, we need to quantify the color change that occurs. To understand how we quantify color, let us first review what color is. White light is a mixture of all the visible wavelengths of light. Objects will absorb some wavelengths of light and transmit (or let pass) other wavelengths depending on the molecule's energy levels (see figure 1).

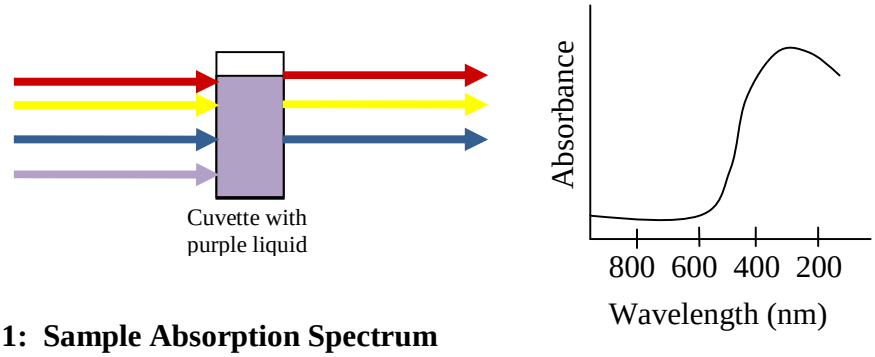


Figure 1: Sample Absorption Spectrum

In our case, as the GAG reacts, it will turn pink and if there is a large amount of GAG, the reaction will appear purple. To measure how pink or purple the sample is, the solution is hit with a specific wavelength of light (525 nm), and the amount of light that is absorbed by the sample is measured. You will use a plate reader to take this reading. One advantage of a plate reader is that it is high-throughput, meaning it allows many samples to be analyzed quickly.

Safety Information

One of the most important responsibilities of a chemical engineer is safety. Safety is a number one priority in a chemical plant or a research lab. Knowing how to use the personal protective equipment (PPE) and the dangers of the chemicals used are essential to maintaining a safe workplace. This project requires gloves, so you will be trained in how to properly remove them without contaminating yourself or others. In addition, Material Safety Data Sheets (MSDS) are provided in the lab so that everyone knows the risks associated with the materials they work with. This lab is no different. The MSDS for every chemical in the lab are stored in the front of the lab. If you are unfamiliar with a chemical you should either ask someone who works with the chemical what the safety risks are, or you should read the MSDS for yourself.

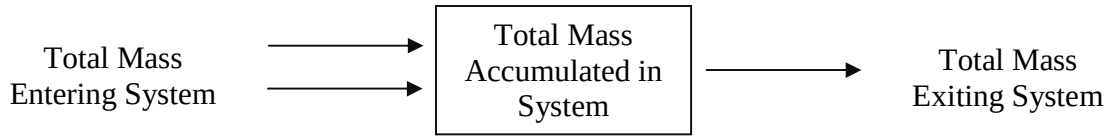
In this lab we will be using two chemicals you should avoid getting on your skin: 1,9 – dimethylmethylene blue and hydrochloric acid. To minimize skin exposure you should wear pants and closed toed shoes as well as a lab coat and gloves. In general, if you do not want a chemical on your skin, you do not want it in your eyes. For this reason you should also wear safety glasses or goggles. If this protection should fail, you should immediately wash the affected area with a lot of water.

We will also be using chondroitin sulfate and glycine which are not as dangerous as 1,9 – dimethylmethylene blue or hydrochloric acid but may cause mild irritation if exposed to your skin. Both of these are powders, and should not be inhaled. If either is exposed to your skin, you should rinse with water.

Calculating the Concentrations of our Prepared Samples: A Look at Material Balances

Chondroitin sulfate is a type of GAG found in cartilage, so we can measure its concentration in solution using the dye described above. Chondroitin sulfate solutions are made by dissolving chondroitin sulfate in water. The stock solution is too concentrated and must be diluted by adding water before analysis.

The first thing that you learn in any Chemical Engineering program is how to balance material (mass) within a process. This is based on the **law of conservation of mass**. The law states that matter can neither be created nor destroyed (see diagram below). Let's do a mass balance on the process describing the dilution of our chondroitin sulfate solution. Mass is conserved in all chemical processes, so the mass of chondroitin sulfate added is equal to the mass of chondroitin sulfate in the final solution.



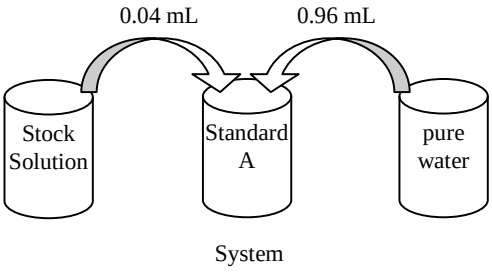
In other words, a material balance on a system states the following:

Total Mass Accumulated = Total Mass In – Total Mass Out

In this case, the system is the container in which the samples are diluted. We will set up material balances describing the dilution of a chondroitin sulfate solution. Chondroitin sulfate solution and water are added to a tube and mixed; there is no mass exiting the system. Your job is to:

1) Set up material balance equations (Accumulation = In - Out)
2) Calculate the chondroitin sulfate solution concentration (µg/mL) of Standard A.

The following schematic shows how Standard A is made:



Unit conversions: 1 mg = 1000 µg 1 mL = 1000 µL

The concentration of the chondroitin sulfate stock solution is 1000 µg/mL.

Now, you will use the material balance procedure to calculate the concentration of Standard A. Since there are two components (chondroitin sulfate and water), you will need to perform a material balance on both to achieve the final goal. The balances will be performed within the system (the tube for Standard A).

1) Material balance for chondroitin sulfate in Standard A
   - *Note*: To calculate the concentration of Standard A, you will examine Standard A before removing any liquid to make the subsequent solutions.

   The material balance for chondroitin sulfate in the system is set up as follows:

   Mass accumulated (µg) = Mass entering (µg) – Mass exiting (µg)

   In this case, nothing is being taken out of the container. Therefore,

   Mass accumulated (µg) = Mass entering (µg) – Mass exiting (µg) = 0

   a. To complete the material balance, you first need to calculate the mass of chondroitin sulfate being added from the stock solution.
      Remember: Mass (µg) = Concentration (µg/mL) * Volume (mL)

   b. Now find the total mass of chondroitin sulfate accumulated.

2) Material balance for water in Standard A
   Calculate the volume accumulated in the system after addition of the stock solution.
   - To simplify the calculations, we will do the calculations in volumes
   - *Note*: Mass is conserved, but volumes are not necessarily conserved. Why can the calculations be done in volumes in this case?

   Volume accumulated (mL) = Volume added from stock (mL) + Volume added from water (mL) – Volume exiting tube (mL)

3) Calculate the chondroitin sulfate concentration (µg/mL) of Standard A.

In Standard A:

Total mass of chondroitin sulfate accumulated =_____ µg

Total volume of water accumulated =_____mL

Chondroitin sulfate concentration

$$= \frac{\text{total mass of chondroitin sulfate}}{\text{total volume of water accumulated}} = \_\_\_\_\_ \mu g/mL$$

Congratulations! These calculations are similar to those done in the first chemical engineering class at Purdue. Now we need to calculate the concentrations of Solutions B, C, D, and E. Solutions B – E were made via a dilution series. To summarize, half of Standard A was mixed in a 1:1 ratio with water to create Standard B. Standard C was made in a similar fashion from Standard B, and so on. We therefore recognize that solutions B, C, D, and E are half the concentration of the previous solution. Standard F is a blank containing no chondroitin sulfate. Fill in the blanks below.

Standard B:
Chondroitin sulfate concentration=_____ µg/mL

Standard C
Chondroitin sulfate concentration=_____ µg/mL

Standard D:
Chondroitin sulfate concentration=_____ µg/mL

Standard E:
Chondroitin sulfate concentration=_____ µg/mL

Standard F:
Chondroitin sulfate concentration= 0 µg/mL

Using your data and calculations, we are now going to construct a standard curve that relates concentration to color (absorbance).

Measuring the Samples

Colorimetric Reaction of GAG:

To determine how much GAG is in your cartilage sample, you will react the sample with a basic dye. GAG is an acidic molecule which means it easily lets go of positively charged hydrogen ions and is negatively charged itself:

$H^+ GAG^- \rightarrow H^+ + GAG^-$

The dye is basic, which means in solution it exists with many hydroxide ions and it is positively changed itself:

$Dye^+ OH^- \rightarrow Dye^+ + OH^-$

When the acid and base react we get a neutralization reaction:

$H^+ + GAG^- + Dye^+ + OH^- \rightarrow$ Dye-GAG complex $+ H_2O$

Now that you have performed the chemical reaction, you need to quantify the extent of the color change. To do this, you will use an instrument called a spectrophotometer. As you learned earlier, color depends on the wavelengths of light an object absorbs. The spectrophotometer measures the extent to which a sample absorbs certain colors of light.

Experimental Procedure

1) Add 20 µL of each chondroitin sulfate standard (samples A-F) to a 96 well plate in duplicate or triplicate. Mark on your plate map the location of the samples. Do the same with your unknown samples.
2) Add 250 µL 1,9-dimethylmethylene blue reagent (DMB, basic dye) to each well (be careful not to bring liquid from one well to another).
3) Measure the absorbance of each sample at 525 nm in the plate reader immediately after adding DMB to each well.
4) Record data in plate map.

NOTE: Be careful not to cross-contaminate wells with tips!

Look at the unknown solutions in your plate. Based on color, which standard solutions are they most similar to? What does this mean?

Data – Fill in sample NAME and ABSORBANCE in each well used

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Data Analysis and Results

1. When taking the absorbance readings of your prepared samples, how much did the measurements vary for each sample? What do you think contributed to this variation?

2. What is the shape of your standard curve? What are the concentrations of your two unknown samples? Include the graphs you used to estimate the concentrations.

3. Looking at your data and analysis, how accurate do you think the concentrations you estimated are? Are there any problems with the data? What changes would you make to improve your results?

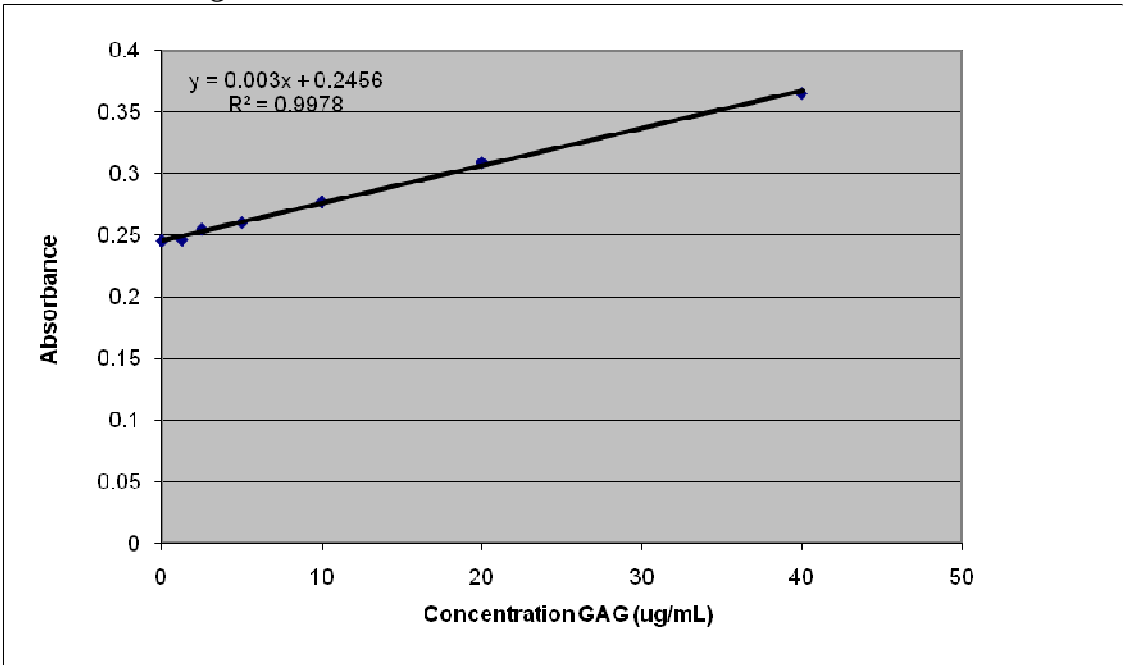
4. We used one of many methods used to characterize cartilage replacements. Besides the methods discussed today, name two other measurements or experiments you think might be important for a fully functional cartilage replacement.

For instructors only

Q1: The most probable cause of variation in these experiments is pipetting error.
Q2:

Calibration curve for:

Standard A = 40 ug/mL
Standard B = 20 ug/mL
Standard C = 10 ug/mL
Standard D = 5 ug/mL
Standard E = 2.5 ug/mL
Standard F = 0 ug/mL



Unknown samples will be:

Sample 1 : 15 ug/mL
Sample 2: 80 ug/mL

Q3: One of the samples will lie outside of the calibration curve so there is a question of whether it lies in the linear regime for this assay. The reaction does saturate at very high concentrations. There are at least two possibilities to fix the experiment. If the unknown sample is not saturated, you could create another standard concentration that encompasses the unknown. Otherwise, the unknown sample of high concentration can be diluted.
Q4: Some examples – mechanical characterization, immune rejection studies, etc.