LAB MODULE 5: ***Histological Examination of Biomaterials Implanted in vivo***

In this lab students will learn about histological staining and examination of cells and tissues. This lab will focus on the cells prevalent in the foreign body response such as neutrophils, macrophages and foreign body giant cells, as well as cells involved in the bone healing process. Students will use microscopy techniques to investigate the body's response to surface eroding polyanhydrides that were implanted *in vivo* in a murine femoral defect model.

REFERENCES:

1. Kim, J., et al., *Osteoblast growth and bone-healing response to three-dimensional poly(ε-caprolactone fumarate) scaffolds.* J Tissue Eng Regen Med, 2011.

PRE-LAB REQUIREMENTS:

1. Print, read and understand the histology document written by the graduate students. This information will be necessary for an understanding of the tissues examined in this laboratory and will be a useful supplementary material during the laboratory period.
2. Read *Kim et al. 2011* in preparation for a brief quiz.

BACKGROUND:

The femur models examined in this laboratory come from an experiment performed at the University of Rochester to promote bone regeneration in mice. Mesenchymal stem cells (MSCs) promote bone growth at the site of fractures and defects in long bones; however MSCs have a tendency to migrate away from fracture sites due to the native SDF-1α gradient. Pepducins, a category of cell-penetrating peptides have been shown to promote stem cell mobilization and improve chemoattractant effects. A surface eroding polyanhydride (SEP) delivery system was developed to provide local, programmable, and sustained (1-5 days) release of pepducin to MSC, providing chemoattractant cues to enable/enhance bone tissue regeneration

In this experiment, mice were given 4 mm diameter femoral defect, and then injected with MSCs via the tail (day 0). The defect site was then treated with the SEP being developed, and the resultant bone healing process tracked over three weeks. Three different treatment groups are discussed in this lab, each of which involves delivery of pepducin at the site of the induced defect. The specific pepducin used in this experiment is ATI-2341, a CXCR-4 agonist. In order to control the release kinetics of pepducins at the site of the femur defect and thus control the regeneration of bone tissue, two SEPs with different degradation properties were tested. By changing the degradation properties of the SEPS, the rate of pepducin release and allowed bone ingrowth was varied.

Each group will examine histological samples from hree treatments for femoral defects over a 21 day healing period. The three treatments applied to the femoral defect are (1) slowly degrading polyanhydride loaded with pepducin in the defect site, with concurrent tail vein injection of mesenchymal stem cells (MSCs), (2) quickly degrading polyanhydride loaded with pepducin in the defect site, with concurrent tail vein MSC injection, and (3) delivery of pepducin in a saline solution to the defect site with concurrent tail vein MSC injection. Each of these treatments was applied immediately after creation of the femoral defect, and histological sections were collected 3, 7, 14, and 21 days later. For each time point and treatment, a section has been stained with (1) Hematoxylin and Eosin (H&E) and (2) Alcian Blue and Orange G (A&O).

MATERIALS:

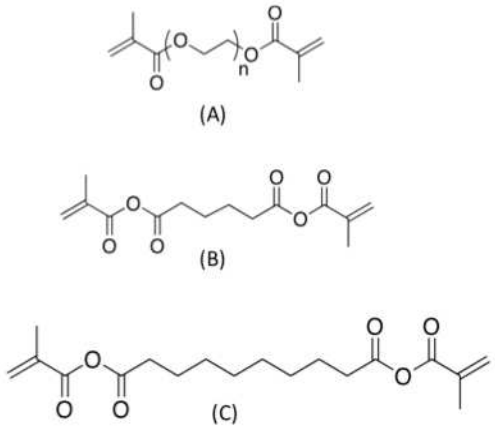


Figure 1. (A) Dimethacrylated poly(ethylene glycol), (B) adipic acid methacrylate and (C) sebacic acid methacrylate

The two surface eroding polymers (SEPs) are block copolymers composed of (A) dimethacrylated poly(ethylene glycol) (PEGDM), (B) adipic acid methacrylate and (C) sebacic acid methacrylate. The slowly degrading SEP is composed of 75 wt% sebacic acid methacrylate and 25 wt% PEGDM before adding pepducin and photoinitiator. The quickly degrading SEP is composed of 50 wt% adipic acid methacrylate and 50 wt% PEGDM. The degradation of each SEP is dependent upon breaking the ester groups in each block copolymer.

PROTOCOL:

1) Each group will examine slides for each treatment at four different time points with two different stains. The labels on the slides correspond to the treatment and time since femoral defect as seen below:

Treatment	Day 3	Day 7	Day 14	Day 21
Slowly Degrading + Pepducin + MSC	ES 2053	ES 2077	ES 2098	ES 2120
Quickly Degrading + Pepducin + MSC	ES 2060	ES 2083	ES 2102	ES 2123
Saline Delivery of Pepducin + MSC	ES 2065	ES 2087	ES 2109	ES 2130

   Every slide also has a number between 1 and 10 in the bottom right corner of the label. 1 to 5 indicates the section is stained with H&E. 6 to 10 indicates the section is stained with A&O. Each lab group should have a total of 24 slides to image

2) Each slide should be photographed for the lab report and post-lab analysis. Be sure to focus on the site of the femoral defect. It will be important that you keep track of the treatment group, stain, and time point in each image so that you can properly present them in the report. The use of 10x magnification is recommended; however, higher magnification should be used to investigate areas of particular interest. An image of a

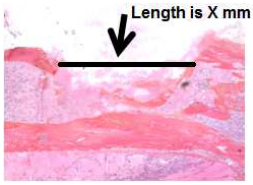
hemocytometer should also be collected at each magnification used so that scale bars can be added to the images.

3) During lab, for each sample investigated, undergraduate students will fill out the histological interpretation worksheet. These worksheets were designed by the graduate students who will be aiding the undergraduates in their interpretation of the histological images. These sheets will be submitted as "Data & Observations" with the lab report and will be graded.

POST-LAB:

Once the in lab requirements are complete students are required to write a formal lab report summarizing their experimental process and presenting their findings. In addition students must answer the following questions, making sure to refer back to their findings summarized in the data collection worksheets:

1) Using ImageJ, determine the cross-sectional area of the femoral defect for each treatment at each day. Assume the histological section is in the center of the femur, and the wound area can be approximated by a circle. Each member of your group is responsible for measuring the CSA, then inputting the result into the google doc. so the class may use the pooled data. Note: as the H&E and A&O stains were performed on serial slices, only one stain for each treatment/time condition needs to be measured. As the lab is due 5/7, this data needs to be posted by midnight on 4/23.



CSA of Defect = $\pi (X/2)^2$

2) Graph cross-sectional area of the defect vs. time for each treatment on a single plot. Include a day 0 theoretical "data" point (the defects were created using a 4 mm drill bit). Explain any trends. Are these measurements consistent with the expected results?

3) When does the inflammatory response appear for each treatment? Which treatment group(s) promotes a foreign body response? Why?

4) What type(s) of cells are recruited to the site of each implant? Which are involved in wound healing and which are involved in the foreign body response?

5) Describe the changes in organization of bone tissue that has been regenerated for each treatment group. How does the structure of the newly formed bone change during its maturation?

6) Discuss differences in features that are highlighted by H&E staining compared to A&O staining in the distal femur model.

7) Discuss the use of control treatments in this experiment. Is saline delivery of pepducin a true control group? Is there a better control, and what might you expect to see in this control?

8) Based on the histological results over the 21 day recovery period, which treatment most successfully promotes osteogenesis? Which treatment is the least successful? Why is that treatment the least successful?

9) Based on your findings, would you recommend any of the treatments be used clinically to aid in bone healing? If yes, which one and why? If no, why not, how would you modify the system to improve it?