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Devlopment Toward Fluorescence Imaging of Microdialysis Sampling Diffusion Profiles

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DEVELOPMENT TOWARD FLUORESCENCE IMAGING OF MICRODIALYSIS SAMPLING DIFFUSION PROFILES

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By

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1. ABSTRACT

Microdialysis is a diffusion-based method used to collect analytes from specific locations in the body. Matrix Metalloproteinases (MMPs) are important enzymes located throughout the body that play a role in the breakdown of extracellular matrix. Microdialysis sampling in conjunction with appropriate analysis methods can be used to observe the reactions of MMPs in real time. This project is aimed at determining the activity of porcine pancreatic enzyme, a known substitute for MMP-12, using nanodrop spectroscopy. This is a necessary pre-requisite toward using microdialysis in an in vivo imaging setup. These experiments were performed using Succinyl$(Ala)_3$-p-nitroanilide (suc$(Ala)_3$-*p*-NA), 4-nitroaniline, and porcine pancreatic elastase. UV-Vis spectroscopy was a suitable measuring device, since suc$(Ala)_3$-*p*-NA and 4-nitroaniline have maximum absorbance separated by 65 nm. The 100kDa molecular weight cutoff (MWCO) probe allowed for the enzyme to diffuse into the collection vials, causing elevated recovery of the p-nitroaniline. This meant that a 20kDa MWCO membrane had to be used. As with most microdialysis, at slower flow rates, the collection of p-nitroaniline increased, collecting 49.5 μM at 1μL/min and 18.7 μM at 5μL/min using the 20kDa MWCO probe. Using UV-Vis it was possible to eliminate the overlapping absorbance values of the suc$(Ala)_3$-*p*-NA and p-nitroaniline.

2. Introduction

2.1 Overview of Experiment

This research project will primarily focus on the enzyme porcine pancreatic elastase and the substrate succinyl$(Ala)_3$-p-nitroanilide. The beginning of this project focused on the extraction efficiency of Succinyl$(Ala)_3$-p-nitroanilide through water. After this, the reaction with porcine pancreatic elastase at various flow rates to determine the optimal flow rate. The ultimate goal of this experiment is to be able to observe the reaction of Succinyl$(Ala)_3$-p-nitroanilide or other suitiable substrates with porcine pancreatic elastase via fluorescent imaging.

2.2 Microdialysis Sampling

A. Basics of Microdialysis Sampling

Microdialysis Sampling is a diffusion-based method used to collect analytes from specific locations in the body. Microdialysis sampling generally consists of four parts: a syringe, syringe pump, microdialysis probe, and a vial to collect dialysate. The microdialysis probe has two pieces of tubing, one of which is the inlet and the other is the outlet, and also the membrane, which is the most important part of the probe. A perfusate is loaded into the syringe and is pumped through the inlet and diffuses through the semipermeable membrane of the probe. The analyte then diffused back through the semipermeable membrane and travels through the outlet tubing and is collect in a collection vial. The dialysate that is collected can then be quantified to determine its concentration.

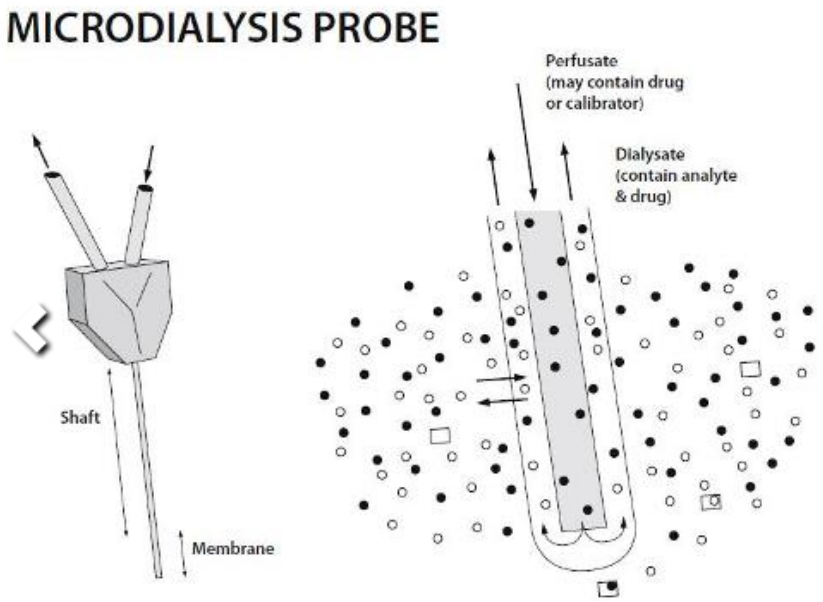

Figure 1: Example of Microdialysis Sampling and Diffusion around membrane.[1]

Originally microdialysis sampling was developed to obtain real-time chemical information from rodent brains. [2] This is because microdialysis probes are small, having a diameter of about 200-500 μm and a length between 1 and 30 mm.[2, 3] Along with this, microdialysis probes have two distinct advantages the classic sampling techniques lack. These are being able to use one animal and keeping them alive throughout the experiment, and also being able to continuously monitor chemical changes in the subject.[3] Now, microdialysis sampling has been used in almost every organ.[2]

B. Quantification of Microdialysis Sampling

After collecting the dialysate using microdialysis sampling, it then has to be quantified. This project primarily focuses on the extraction efficiency (EE), or more specifically the relative recovery (RR). The recovery describes the overall mass transport of a substance to and from the microdialysis probe.[4]

$$EE = \frac{C_{outlet} - C_{inlet}}{C_{sample} - C_{inlet}} \quad (1)^{4}$$

In this equation, C_{outlet} is the concentration of the microdialysis outlet tube (the dialysate), C_{inlet} is the concentration of the inlet tube (the perfusate), and C_{sample} is the analyte concentration far away from the microdialysis probe.[4] In this project C_{inlet} is generally 0, so the extraction efficiency can be simplified to the relative recovery.

$$RR = \frac{C_{outlet}}{C_{sample}} \quad (2)$$

Microdialysis sampling recovery is susceptible to many different factors. The first of which is the flow rate of the perfusate. Typically, flow rates are between 0.5 and 2μL/min.[2] In this experiment, flow rates will be as high as 5μL/min. Typically, the higher the flow rate the lower the relative recovery will be. This is illustrated in figure 2 below. Another factor that will affect the recovery is the size of the molecule being collected. The larger the molecule is, the more difficult it is to diffuse across the microdialysis membrane. This is because the semipermeable membrane on the probes typically has a molecular weight cutoff. The probes used in this experiment have a cutoff of 100kDa. This will not affect the recovery in this experiment, as the analyte being collected is much below this limit.

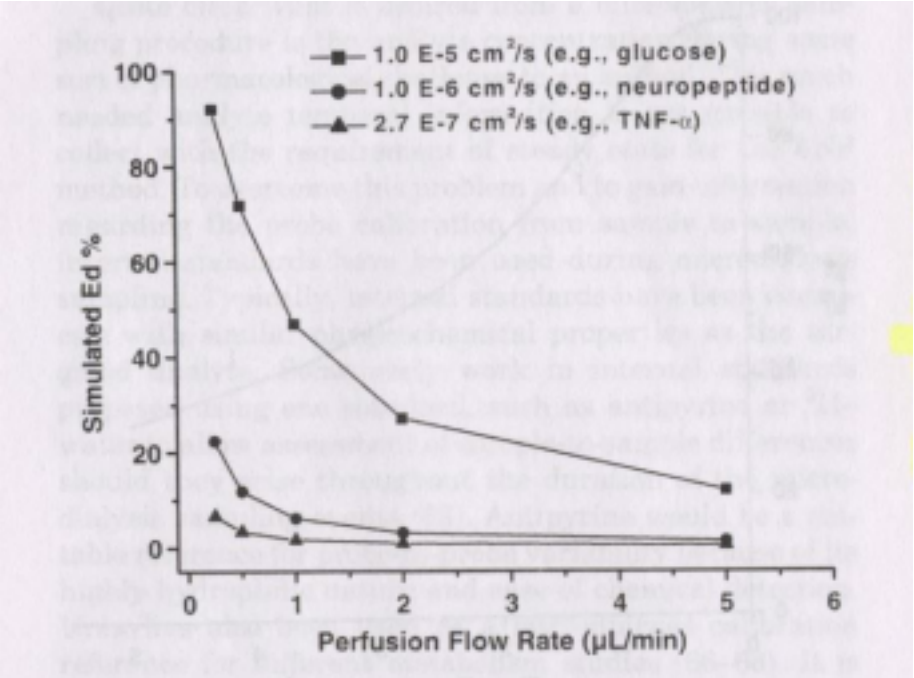

Figure 2: Simulated Extraction Efficiency of molecules with different molecular weights. Displays that molecules with lower molecular weights have higher EE and also that slower flow rates yield a higher EE.[2]

2.3 Matrix Metalloproteinases

Matrix Metalloproteinases (MMPs) are a subfamily of zinc- and calcium-dependent enzymes belonging to the metzincin superfamily.[5] All MMPs play a vital role in the breakdown of extracellular matrix (ECM). The breakdown of this matrix is important in biological functions, such as, angiogenesis, embryonic development, and wound healing, and also pathological functions, such as, arthritis, cancer, and cardiovascular disease.[4]

This project will primarily focus on porcine pancreatic elastase as a mimic for MMP-12. MMP-12 is also known as neutrophil elastase.[6] This enzyme is commonly found in the lungs and breaks down several extracellular matrix proteins. One of these proteins is elastase.[7] An experiment was performed on mice made genetically

deficient of MMP-12. In this experiment the mice with less MMP-12 developed emphysema less than the normal mice.[7] Along with this, cigarette smoke is known to enhance the activity of MMP-12, becoming the only macrophage elastase present in the lungs.[7] By finding the activity of MMP-12, further research to develop an inhibitor of this elastase can be developed. In this project an inexpensive alternative to MMP-12 will be porcine pancreatic elastase, as it cleaves the same substrate as MMP-12.

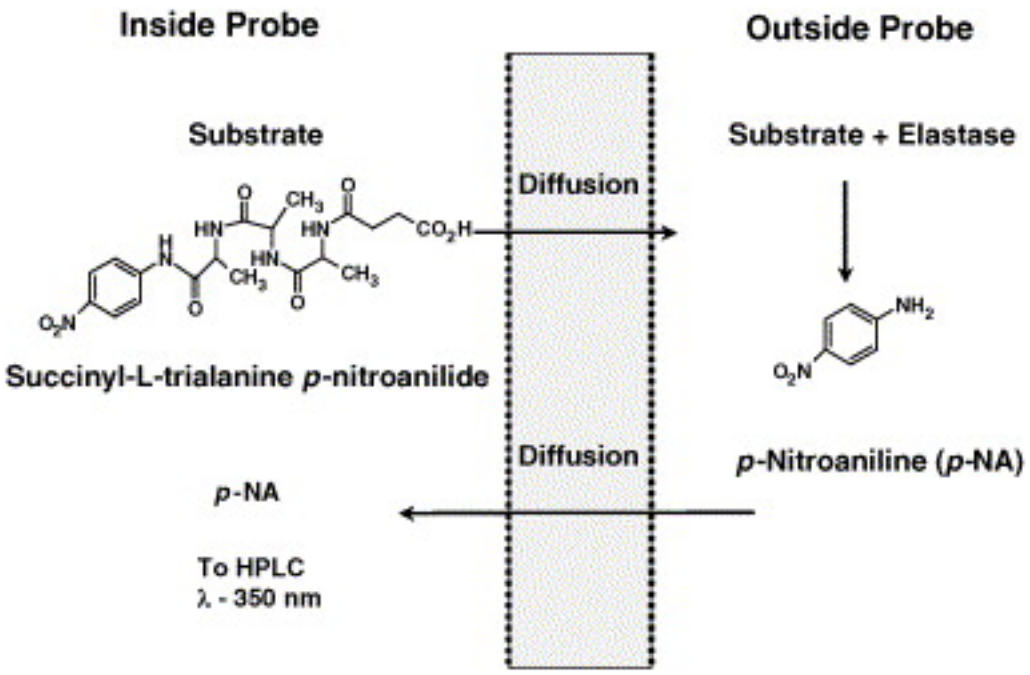

Figure 3: Example of MMP-12 cleavage of substrate being used, Suc(Ala)3-p-NA.[4]

2.4. Nanodrop Spectroscopy

This experiment uses Nanodrop Spectroscopy instead of traditional spectroscopy or other measuring techniques. Similar research has been done which

used Hitachi UV spectrometer.[4] Nanodrop Spectroscopy however has been shown to be more accurate in different studies however.[8]

Table 1: Statistical Experiment between Hitachi Spectroscopy vs. Nanodrop.

Table 2. Statistical Summary of 4-Method Comparison

method	mean concentration (μg/mL)	standard deviation	% difference[a]	CV %	*t*-value	*t*-crit	*P*-value
(1) U-2000 Spec (Hitachi)	72.84	2.89	+38.08	4.0	−41.76	2.03	1.89×10^{-31}
(2) ND-1000 spec (NanoDrop)	67.92	4.71	+28.77	6.9	−19.32	2.03	3.00×10^{-20}
(3) PicoGreen (Molecular Probes)	48.44	1.67	−8.17	3.5	+15.47	2.03	3.20×10^{-17}
(4) Quant-iT (Molecular Probes)	58.59	5.39	+11.07	9.2	−6.51	2.03	1.66×10^{-7}

[a] From the assigned ICP-OES value.

Along with this there are distinct advantages to the use of Nanodrop compared to the Hitachi. One such advantage is the small amount of analyte that the Nanodrop needs to obtain a proper reading. In this experiment 2µL of dialysate were used to conduct the absorbance readings. Some other experiments can use as little as 1µL of analyte.[9] This small amount of dialysate is desirable when using microdialysis sampling because the process can be very slow, and obtaining 100µL of sample to perform one absorbance test would not be practical. This experiment will use the same process as earlier research to determine if the Nanodrop Spectroscopy will yield similar results to the earlier research done using the Hitachi Spectroscopy.

In spectroscopy, all substances have a maximum absorbance value. This is the point where the substance will absorb the most light. Substances still absorb light at wavelengths other than their maximum wavelengths however. Since succinyl$(Ala)_3$-p-nitroanilide and 4-nitroaniline's maximum absorbance are only separated by 65 nm, there absorbance's will overlap. Below is an equation that

gives an example of how this overlap will affect the absorbance of multiple substances.

$$A' = \varepsilon'_x b[X] + \varepsilon'_y b[Y]$$

$$A'' = \varepsilon''_x b[X] + \varepsilon''_y b[Y] \quad (3)^{10}$$

In this equation, A' is the absorbance at wavelength 1 and A'' is the absorbance at wavelength 2. Along with this, ε'_x is the absorptivity of x at wavelength 1, ε'_y is the absorptivity of y at wavelength 1, ε''_x is the absorptivity of x at wavelength 2, $\varepsilon''_y b$ is the absorptivity of y at wavelength 2, b is the pathlength, [X] is the concentration of X, and [Y] is the concentration of Y.[10] This equation demonstrates how having multiple compounds in a solution will cause an elevated absorbance values. This overlap can be accounted for by using the following equation.[10]

$$[X] = \frac{\begin{vmatrix} A' & \varepsilon'_y b \\ A'' & \varepsilon''_y b \end{vmatrix}}{\begin{vmatrix} \varepsilon'_x b & \varepsilon'_y b \\ \varepsilon''_x b & \varepsilon''_y b \end{vmatrix}} \qquad [Y] = \frac{\begin{vmatrix} \varepsilon'_y b & A' \\ \varepsilon''_y b & A'' \end{vmatrix}}{\begin{vmatrix} \varepsilon'_x b & \varepsilon'_y b \\ \varepsilon''_x b & \varepsilon''_y b \end{vmatrix}} \quad (3)^{10}$$

In this equation, A' is the absorbance at the maximum wavelength of one substance and A'' is the maximum absorbance of the other substance in solution. $\varepsilon'_x b$ is the matrix of coefficient of substance x at wavelength A', while $\varepsilon''_x b$ is the matrix of coefficient of substance x at wavelength A''. $\varepsilon'_y b$ is the matrix of coefficient of substance y at wavelength A', and $\varepsilon''_y b$ is the matrix of coefficient of substance x at wavelength A''. In this equation, each symbol $\begin{vmatrix} a & b \\ c & d \end{vmatrix}$ is a determinant. This is a shorthand way of writing the product is $a\ x\ d - b\ x\ c$.[10]

3. Materials and Methods

3.1 Chemicals

Succinyl$(Ala)_3$-p-nitroanilide, 4-nitroaniline, and porcine pancreatic elastase were all obtained from Sigma (St. Louis, MO). Elastase and succinyl$(Ala)_3$-p-nitroanilide solutions were prepared daily. The microdialysis probes used were CMA/20 probes and CMA/12 obtained from CMA/Microdialysis (North Chelmsford, MA, which have a molecular weight cutoff of 100kDa and 20kDA respectively. The pump and syringe used were obtained from BASi (West Lafayette, IN). The syringe used was a 1mL syringe.

3.2 Determining maximum absorbance

To determine the maximum absorbance of succinyl$(Ala)_3$-p-nitroanilide and 4-nitroaniline 100 μM solutions were made. The succinyl$(Ala)_3$-p-nitroanilide was made using 0.1M sodium phosphate buffer, pH 7.1. The 4-nitroaniline solution was made using 95% ethanol. These solutions then had their absorbance observed in the Thermo Scientific Nanodrop 2000c spectrometer (Waltham, MA) in a range of 350-400nm for 4-nitroaniline and 280-350nm for succinyl$(Ala)_3$-p-nitroanilide. The ranges were tested in the nanodrop machine.

3.3 Microdialysis sampling

Microdialysis sampling was performed using a CMA/20 microdialysis probe with a 100kDa molecular weight cutoff. The perfusion fluid contained 500 μM succinyl$(Ala)_3$-p-nitroanilide in 0.1M sodium phosphate buffer, pH 7.1. First, the substrate was perfused through HPLC grade water to determine the recovery of just

the substrate. This means that the perfusion fluid was a 500μM solution of the substrate and the probe was immersed in HPLC water. The microdialysis probes were perfused at varying flow rates of 1μL/min-5μL/min, in increments of 1μL/min. Each sample was collected in triplicate in three separate 0.5 mL vials. Since the Nanodrop only requires 2μL of a sample to make a measurement, only 4-5μL of sample were collected in each vial. The extra perfusate was collected in case of accident. Each of the vials were weighed before and after collecting the sample. This was to ensure that the correct amount of perfusate was collected. By taking the initial mass of the vial and subtracting it from the mass after collection, the volume of the sample was found. Between each flow rate, the microdialysis probe was equilibrated for approximately 15 minutes. This is to insure that there is no carryover in the collection tube of the probe. During this time period, all products were allowed to collect in a disposal vial. Along with this, the probe was immersed in a fresh vial of water for every flow rate. The elastase was subject to the same procedure and was prepared at 0.5 units/mL in the same 0.1M sodium phosphate buffer, pH 7.1. A fresh elastase solution was used for each varying flow rate.

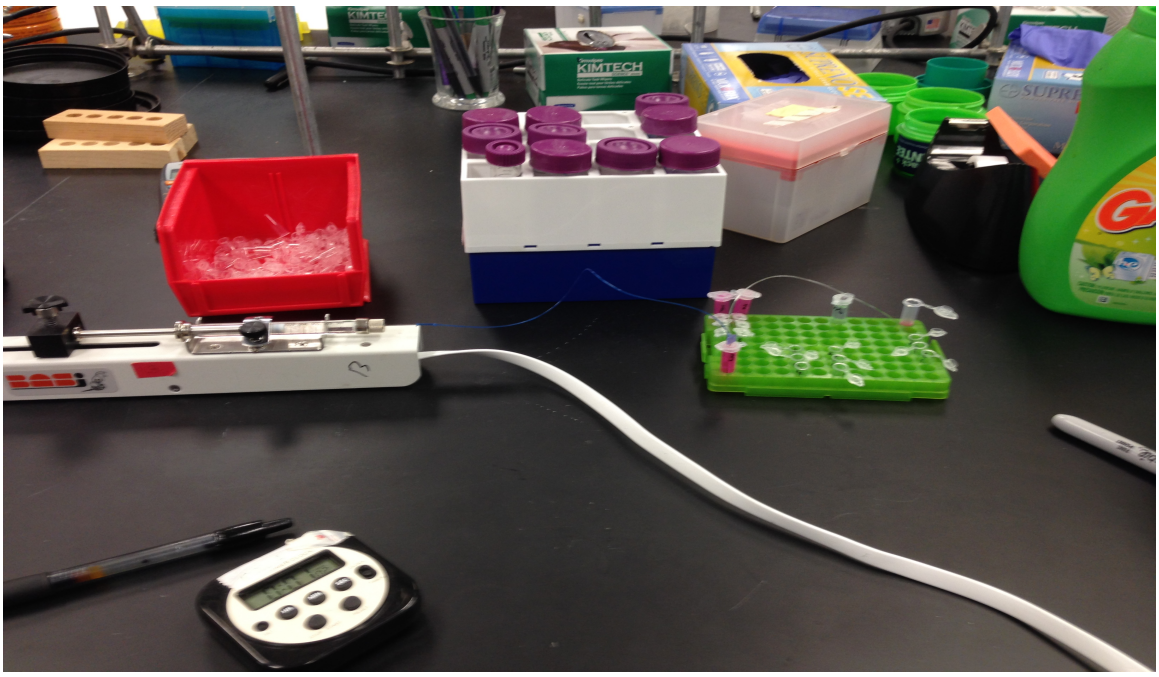

Figure 4: Overview of In-vitro set-up. Pump on left pushes perfusion fluid at set flow rate through syringe and consequently through microdialysis probe.

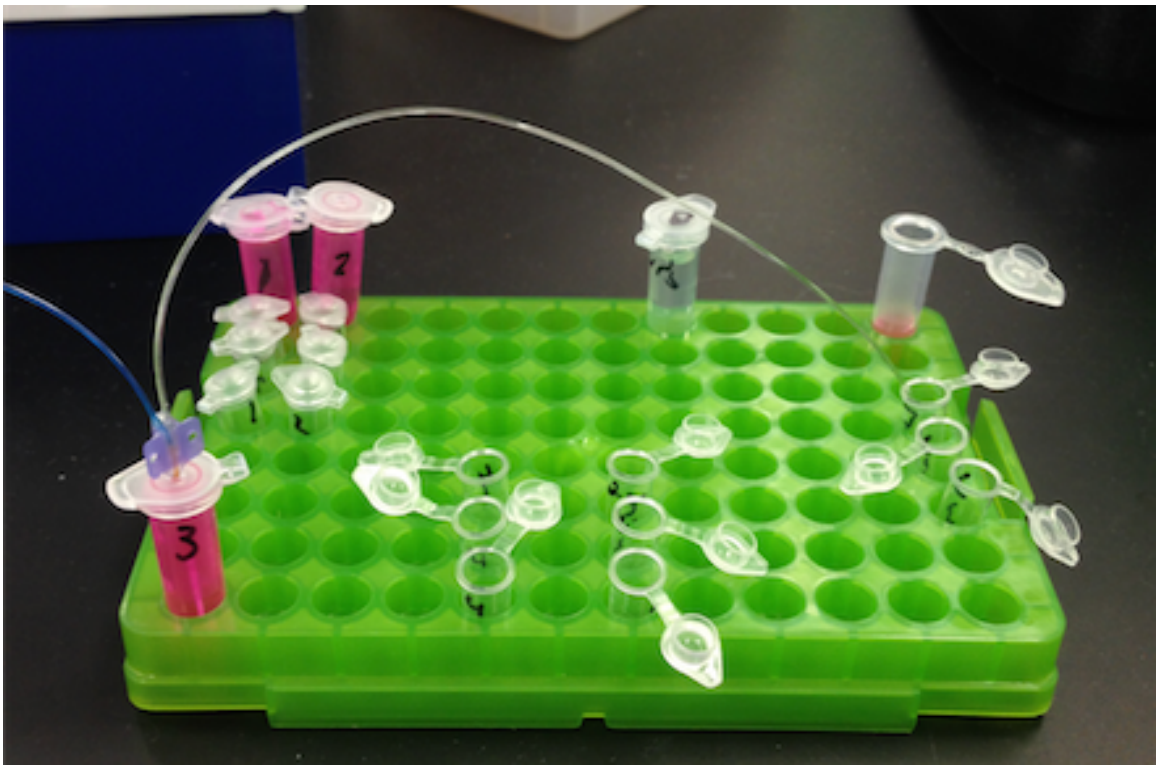

Figure 5: Close-up of experiment. Microdialysis is placed in vial with elastase and perfusate is collected in three separate 0.5 mL vials for each varying flow rates.

3.4 Measurements using Nanodrop Spectrometry

The concentrations recovered from the microdialysis sampling was performed using UV-Vis spectrometry. The optimal wavelength of succinyl$(Ala)_3$-p-nitroanilide was found to be 315nm and for 4-nitroaniline was found to be 380nm. Calibration curves were performed for both succinyl$(Ala)_3$-p-nitroanilide and 4-nitroaniline at their maximum wavelengths. Both of these calibration curves were done using serial dilution. The nanodrop was then used to measure the absorbance of the perfusate collect in the vials from the microdialysis sampling. Each vial was only measured once and the three separate absorbance values were averaged to obtain the final absorbance. This final absorbance was then plotted against the calibration curve to determine the concentration of the analytes in the perfusate. The standard deviation of the absorbance values was also found and plotted against the calibration curve to determine the standard deviation of the concentration.

3.5 Fluorescent Imaging of Microdialysis Probes in Agar Blocks.

For fluorescent imaging, the probes were placed in a 1% agar solution. The agar solutions were made by preparing a 1 mL of TAE 50x buffer solution and diluting this into 50mL of TAE 1x buffer solution. TAE buffer is a solution containing Tris base, acetic acid, and ethylenediaminetetraacetic acid (EDTA). Solid agarose was then added to this solution and heated in the microwave until it was fully dissolved. This was then allowed to cool back down to room temperature to form an agar block. The probe was then inserted into the block, and fluorescent imaging was then performed on the block with a flow rate of 1uL/min.

In order to infuse porcine pancreatic elastase into the agar block, the solution was allowed to cool to around 45°C. The enzyme was then added to the agar solution, and the agar cooled to room temp to form a solid block.

4. Results and Discussion.

The first experiment ran was to determine the wavelength that produced the maximum absorbance for both 4-nitroaniline and succinyl$(Ala)_3$-p-nitroanilide. Figure 6 shows the absorbance spectrum of 4-nitroaniline and demonstrates that the maximum absorbance is at 380nm. Figure 7 shows the absorbance spectrum of succinyl(Ala*)3*-p-nitroanilide and demonstrates that the maximum absorbance is at 315 nm.

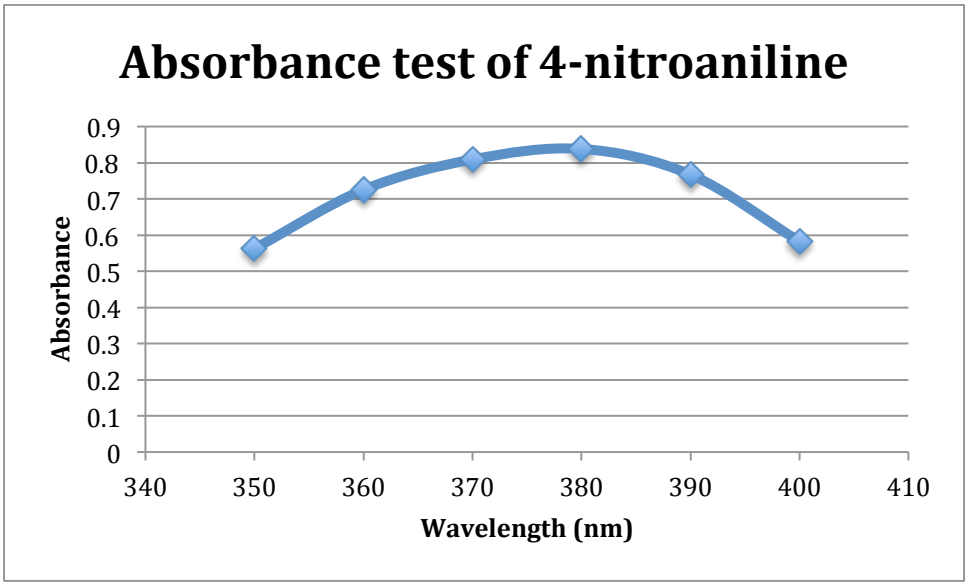

Figure 6: Determining the maximum wavelength of 4-nitroanline. Maximum wavelength was determined to be 380nm.

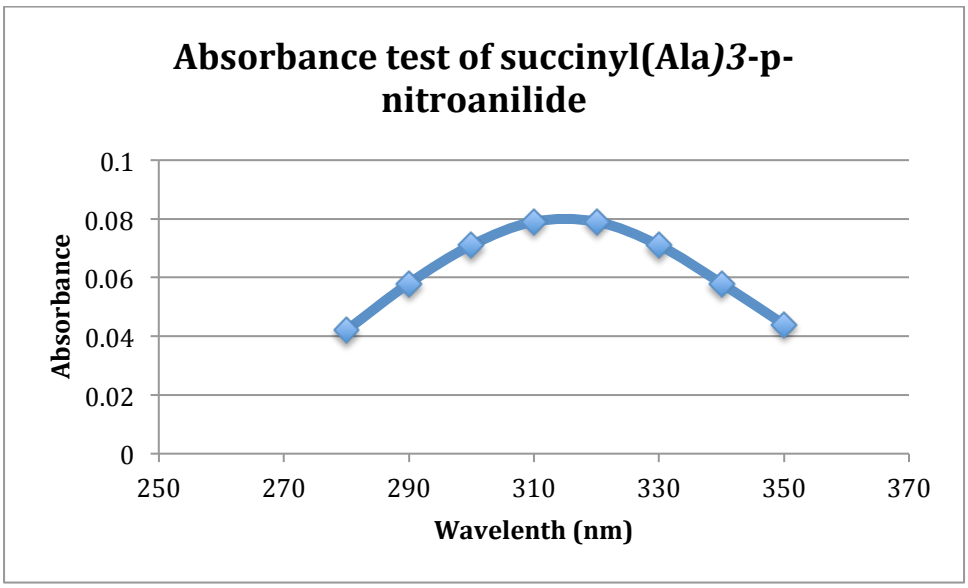

Figure 7: Determining the maximum wavelength of the substrate. Maximum absorbance was found to be 315 nm.

Once the maximum absorbance values were found calibration curves were made for each succinyl(Ala*)3*-p-nitroanilide and 4-nitroaniline. These calibration curves were used to determine the absorbance of the substrate and product at specific concentrations and to obtain the molar absorptivity constant. Figure 8 is an example of a calibration curve of 4-nitroanaline made by a series of dilution. The molar absorptivity constant was 0.0016 $\frac{mol}{Lxcm}$. Figure 9 is an example of a calibration for the substrate, succinyl(Ala*)3*-p-nitroanilide**.** The molar absorptivity of this substrate was 0.001 $\frac{mol}{Lxcm}$.

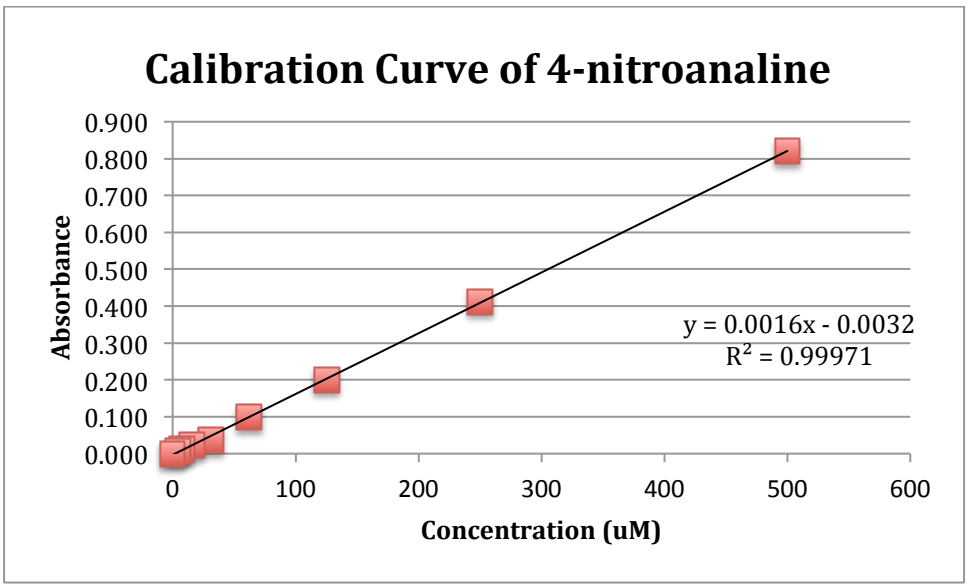

Figure 8: Calibration Curve of 4-nitroaniline. Molar absorptivity is 0.0016.

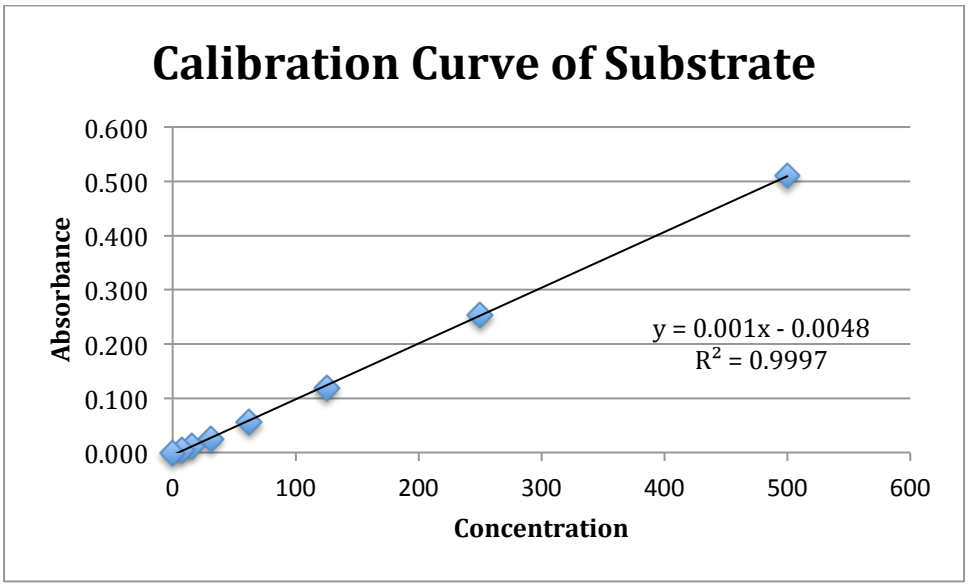

Figure 9: Calibration Curve of succinyl(Ala*)3*-p-nitroanilide. Molar absorptivity is 0.001.

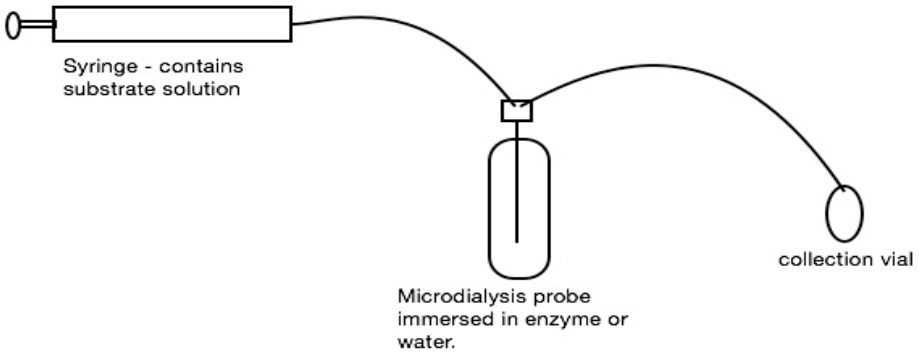

Figure 10: Picture demonstrating where all substances are in experiment.

Figure 10 is a demonstration of what the experimental set up looks like and also where all substrates and enzymes are located. The syringe contains 500 uM succinyl(Ala*)3*-p-nitroanilide in most experiments. The microdialysis probe is submerged in either water or 0.5 units/mL enzyme. The collection vial collects the dialysate.

Once the Calibration Curves were made the substrate was perfused through water to determine the extraction efficiency. Results of this experiment can be seen in table 2.

Table 2: Delivery of 500µM substrate in H2O.

Flow Rate	Concentration (µM)	Percent Recovery
1uL/min	202.800	59.440
2uL/min	294.133	41.173
3uL/min	333.467	33.307
4uL/min	388.800	22.240
5uL/min	410.800	17.840

From table 2 it was determined that the substrate was being retained at higher flow rates. This could have to do with the fact that not all of the substrate was being perfused into the vial and some remained in the probe. This would make it to where mostly the substrate was being collected. The substrate was then perfused through 0.5 units/mL enzyme. The results can be seen in table 3.

Table 3: Concentration Recovery of 500µM substrate and p-nitroaniline perfused through 0.5units/mL enzyme using 100 kDa MWCO probe.

Flow Rate	Concentration of substrate (uM)	Concentration of p-nitroaniline (uM)
1uL/min	133.800 ± 26.255	128.042 ± 14.295
2uL/min	282.467 ± 10.368	99.708 ± 9.638
3uL/min	355.467 ± 7.446	76.792 ± 6.719
4uL/min	394.133 ± 8.586	62.000 ± 4.366
5uL/min	416.800 ± 17.697	52.208 ± 8.070

Table 3 shows that the concentration of the substrate follows what was expected from observing the experiment involving water. However, as the concentration of substrate recovered increases, the concentration of p-nitroanline decreases. This could be due to the face that the substrate is being pumped into the enzyme and extracted so quickly that the enzyme never has a chance to cleave the substrate. This leads to the slower flow rates recovering more of the p-nitroaniline since it does not dilute the enzyme surrounding the probe and most of the substrate inserted in the enzyme will be cleaved.

After this first initial experiment, it was found that the enzyme was being diffused through probe and into the collection vials. This allowed the reaction to continue in the collection vials, and leading to inflated recovery of the cleaved substance, 4-nitroaniline. A 20kDa MWCO probe was then used so that the enzyme

would not diffuse through the membrane. The results of this experiment can be seen in table 4.

Table 4: Concentration Recovery of 500µM substrate and p-nitroaniline perfused through 0.5units/mL enzyme using 20kDa MWCO probe.

Flow Rate	Concentration of substrate (uM)	Concentration of p-nitroaniline (uM)
1uL/min	280.889 ± 10.889	64.292 ± 5.442
2uL/min	390.148 ± 12.026	55.125 ± 4.954
3uL/min	427.185 ± 5.505	48.250 ± 4.366
4uL/min	440.148 ± 5.919	43.458 ± 2.625
5uL/min	448.667 ± 6.147	42.625 ± 2.361

It was observed that the recovery of p-nitroaniline was much lower for this experiment. This indicates that the enzyme was reacting and causing excess results in the 100kDa MWCO probe experiments. Since the absorbance values were so close, they caused slight overlap between the substrate and the desired product. Using equation 3, the absorbance values were taken into account. Table 5 is the same experiment as table 4. The only difference was that equation 3 was used to eliminate the overlap absorbance in table 5. This lead to about a 20 percent decrease in p-nitroaniline at the 1µL/min, and about 50 percent at 5µL/min flow rate. The absorbance of the substrate was much higher at 5µL/min than at 1µL/min, which would lead to the much larger overlap.

Table 5: Concentration Recovery of 500µM substrate and p-nitroaniline perfused through 0.5units/mL enzyme using 20kDa MWCO probe and accounting for absorbance overlap.

Flow Rate	Concentration of substrate (uM)	Concentration of p-nitroaniline (uM)
1uL/min	260.169 ± 10.889	49.492 ± 5.442
2uL/min	374.082 ± 12.026	34.421 ± 4.954
3uL/min	414.831 ± 5.505	25.508 ± 4.366
4uL/min	428.955 ± 5.919	19.802 ± 2.625
5uL/min	438.206 ± 6.147	18.715 ± 2.361

5. Conclusion.

The recovery of p-nitroaniline was highest at the slowest flow rate and lowest at the highest flow rates. Since the substrate and product have such a close maximum absorbance value, some of the absorbance will overlap. This overlap issue was solved by using equation 3. An initial major complication in the research is that 100kDa MWCO probes were used in the experiment. The enzyme only has a molecular weight of about 25kDa, so the probes are allowing the enzymes to diffuse through the membrane and thus react with the substrate in the dialysis fluid which is not the intent or design of the experiment. However, with all this, it is still shown that UV-Vis is a viable method of measuring the collection or product in this reaction. Taking the absorbance from the other substrates into account greatly reduces the amount of product recovered. It does not affect the standard deviation because the standard deviation measures the variance between the triplicate samples. Future experiments will be aimed towards taking fluorescent images of substrates cleaved by porcine pancreatic elastase, leading up to in vivo fluorescent images.

6. References

1. Microdialysis. (September, 2014) Lookfordiagnosis, Web
2. Stenken, J. A. (2006) Microdialysis Sampling, In *Encyclopedia of Medical Devices and Instrumentation* (Webster, J. G., Ed.), John Wiley & Sons, Inc.
3. Stenken, J. A. (1999) Methods and issues in microdialysis calibration, *Anal. Chim. Acta 379*, 337-357.
4. Steuerwald, A. J., Villeneuve, J. D., Sun, L., and Stenken, J. A. (2006) In vitro characterization of an in situ microdialysis sampling assay for elastase activity detection, *J. Pharm. Biomed. Anal. 40*, 1041-1047.
5. Hadler-Olsen, E., Fadnes, B., Sylte, I., Uhlin-Hansen, L., and Winberg, J.-O. (2011) Regulation of matrix metalloproteinase activity in health and disease, *Febs J. 278*, 28-45.
6. Nagase, H., and Woessner, J. F., Jr. (1999) Matrix metalloproteinases, *J Biol Chem 274*, 21491-21494.
7. Hautamaki, R. D., Kobayashi, D. K., Senior, R. M., and Shapiro, S. D. (1997) Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice, *Science (Washington, D. C.) 277*, 2002-2004.
8. English, C. A., Merson, S., and Keer, J. T. (2006) Use of Elemental Analysis To Determine Comparative Performance of Established DNA Quantification Methods, *Anal. Chem. 78*, 4630-4633.
9. Gallagher Sean, R. (2011) Quantitation of DNA and RNA with absorption and fluorescence spectroscopy, *Curr Protoc Neurosci Appendix 1*, Appendix 1K.
10. Harris, D. C. (2007) *Quantitative Chemical Analysis*, Seventh ed., W.H. Freeman and Company, New York.