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Development and Characterization of a Paper Based Analytical Device for Heparin Quantification

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Development and Characterization of a Paper Based Analytical Device for Heparin Quantification

by

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Abstract

Paper-based analytical devices (PAD) have gained popularity in the past ten to fifteen years due to many favorable mechanical properties.[1] They have largely been used to analyze small ionic species, such as Na^+ and Li^+, but little research has been done on their capability of to analyze large polyionic species, for instance the highly sulfonated polysaccharide heparin.[2,3] This study explores adaptation of a traditional optode design to a low cost PAD that employs a smartphone as a detector. Throughout the study variables, such as membrane composition, pH, volume of solution, time, addition of a polymer, use of different cell phone apps, and analytes including both low molecular weight heparin (LMWH) and unfractionated heparin (UFH), were examined. The best assay settings were found to be a membrane that included a 2:1 mole ratio of tridodecylmethyl ammonium chloride (TDMA) and ETH 2412, respectively, and the polymer poly(vinyl) chloride (PVC), a sample volume of .5 μL, and an elapsed time of one minute.

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Introduction

Heparin (Figure 1) is the most widely used anticoagulant and antithrombin agent used in hospitals today.[4] Heparin was first discovered in 1918 by William Henry Howell and since its discovery has become vital in procedures, such as cardiovascular, thoracic and orthopedic surgeries.[5] Heparin is also the most common treatment for patients who suffer from pulmonary embolism (PE) and deep vein thrombosis (DVT).[6] Side effects of heparin can include bruising, bleeding episodes, low blood platelet levels, and in severe cases hemorrhaging, osteoporosis, neuropathy, and hypersensitivity which is why it is important to measure heparin efficiently and accurately. Therefore, a method that is capable of accurately and specifically monitoring heparin levels is essential in clinical care settings.

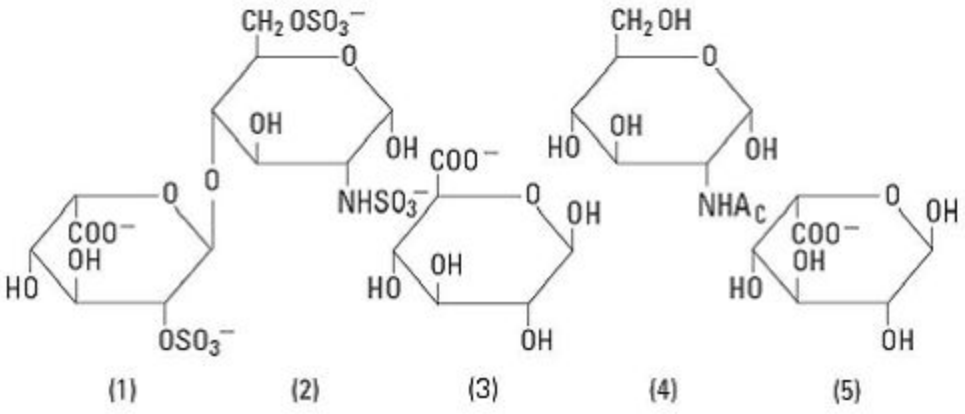

Figure 1. The structure of heparin.[6]

Mechanism of Anticoagulant Activity

The process behind blood clotting in the body is referred to as the Clotting Cascade. It is given this name because for blood to clot there are multiple steps that must happen first before the fibrin and thrombin can form. The process first begins when a person has an injury to the endothelium, the tissue lining the blood vessels and organs. The primary, also known as extrinsic or tissue factor, pathway requires an inactive form of an enzyme to become activated, which then catalyzes the next reaction (Figure 2). Upon the introduction of heparin into the bloodstream it begins to inhibit certain factors of the blood clotting cascade.[7]

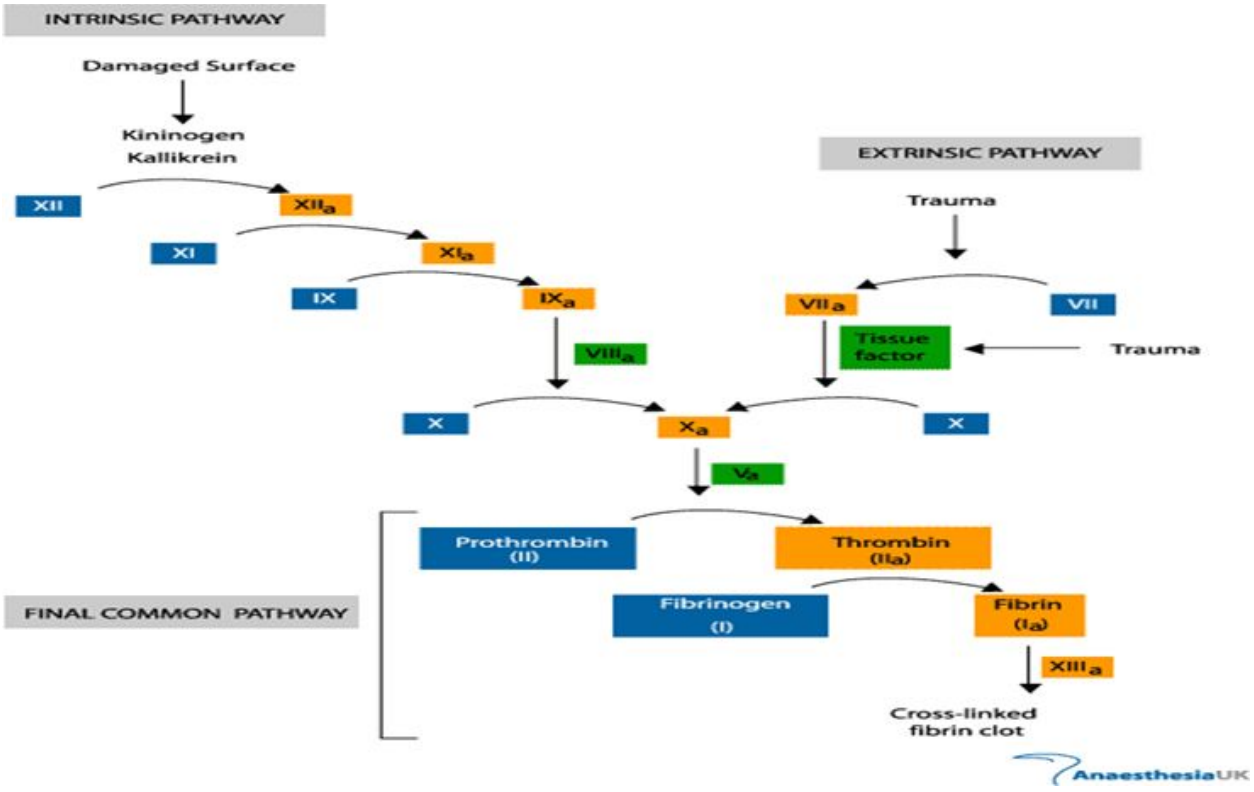

Figure 2. Schematic representation of the blood clotting cascade.[7]

The anticoagulant activity of heparin not only depends on its stereochemistry and orientation of its sulfate residues, but also on its ability to bind to the antithrombin-heparin cofactor AT III (Figure 3). Because of the high negative charge density of heparin, it readily binds to the positively charged lysine sites on AT III causing the cofactor to undergo conformational changes. The conformational changes to AT III makes the arginine groups on it more reactive to the active centers on the serine proteases of thrombin allowing the heparin-AT complex to inactivate thrombin. Only one-third of the administered dose of heparin binds to AT III and at the therapeutic concentrations; the other two-thirds of the dose have minimal to no anticoagulant activity.[8] If the concentrations are higher, then the other two-thirds of the administered heparin will catalyze the AT III effect by activating a second protein, heparin cofactor II. This heparin-AT complex inactivates many of the coagulation enzymes including cofactors IIa, IXa, Xa, XIa, and XIIa.[9] The factors that are the most responsive to inhibition by the heparin-AT complex are IIa and Xa. For the inhibition of factor IIa to occur heparin must bind to AT III and the enzyme at the same time and as a result the heparin molecule must be at least 18 saccharides long, requiring the common pentasaccharide unit and an additional thirteen saccharides.[8]

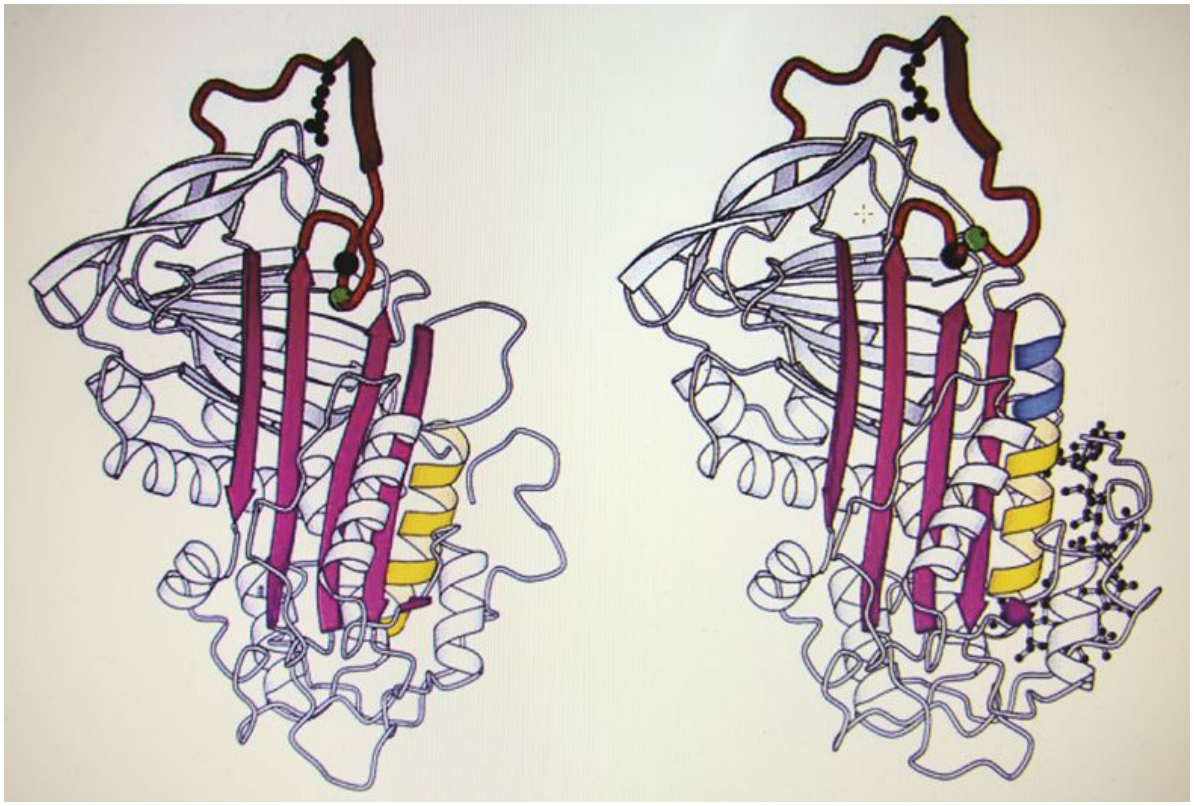

Figure 3. Diagram showing the conformational changes that AT III undergoes when binding to heparin.[10]

Low Molecular Weight Heparin (LMWH) and Unfractionated Heparin (UFH)

The two most common types of heparin include low molecular weight heparin (LMWH) and unfractionated heparin (UFH). LMWH has a mean molecular weight of around 4,000-5,000 daltons (Da). In comparison to UFH mean molecular weight of 12,000 Da. LMWH inhibits Factor Xa (figure 2) in the coagulation cascade by causing a conformational change to AT III.[11] UFH binds more effectively to the coagulation factor IIa. The therapeutic ranges for LMWH and UFH differ slightly from 0.6-1.0 U/mL and 0.3-0.7 U/mL respectively. The complete clinical range for heparin can go as high as 8 U/mL for certain procedures, such as gastrointestinal procedures.[12] Even though LMWH reacts less with platelets than UFH making it less likely to

cause bleeding episode and immuno-allergic thrombocytopenia, it is still important to have accurate monitoring of the platelets levels because such episodes can occur.[13]

Methods for analyzing heparin

Several methods have been developed to measure heparin. One of the most widely used in clinical laboratories is the Partial Thromboplastin Time (PTT).The effectiveness of the PTT test results depends on two coagulation factors Factors XII and fibrinogen. The PTT measures how long it takes for a drop of a person's blood on a slide to show resistance to being pricked with a needle, which is then correlated with the amount of heparin that a person has in their body. However, this is an indirect method to determine heparin since other variables can affect the clotting time. For example, if a person is administered fluids before the PTT test is performed, the clotting time will take longer. Other factors that can affect this test include liver disease, temperature fluctuations and pH. These can lead to false interpretations about the levels of heparin present. If the heparin levels are underestimated and a physician gives them a higher dosage, this can cause the patient to bleed out. PTT also suffers from not being able to measure low molecular weight heparin, such as lovenox, which is sometime given as an alternative to unfractionated heparin.[14]

Another common assay used for heparin determination is the factor Xa assay. This assay exploits the ability of heparin to inhibit Factor Xa. In the Factor Xa assay, the conversion of a colorimetric substrate is affected by the amount of Factor Xa available; this color change is observed with a spectrophotometer. Similar to the PTT assay, the Factor Xa assay is an indirect method of determining heparin and, thus, suffers from similar issues. Additionally, because it is a

colorimetric assay, plasma rather than blood must be used. This increases the sample preparation time.[16]

Additional colorimetric methods have also been developed to measure heparin. The Farndale assay analyzes the shift in the maximum absorbance wavelength of the dye 1,9-dimethylmethylene blue after it binds to the sulfate groups on the heparin molecule. Similar to the Factor Xa assay, this has the disadvantage of needing plasma, rather than whole blood, for analysis.[17]

Electrochemical methods are also commonly used to determine the concentration of heparin in the complex blood matrix. This methods include differential pulse voltammetry (DPV), also known as differential pulse polarography (DPP), linear sweep voltammetry (LSV), and cyclic voltammetry (CV) and potentiometry. Potentiometric polyion-sensitive electrodes have been used to measure heparin in whole blood when the analyte is titrated with its antidote protamine. Methods that exclude the use of titrations would prove easier to use.[12,18]

Paper-based analytical devices

This study explores the development of a low cost paper-based analytical device for heparin that employs a smartphone as the detector. Paper-based analytical devices (PADs) have become popular methods for quantification in the chemistry field over the past ten to twenty years. These paper sensors can be used as a detector for both optical and electrochemical studies. The popularity of PADs include their biocompatibility, biodegradability, time taken to analyze a sample, and many other advantageous that are summarized in Table 1. Paper-based devices are also easy to produce making them accessible world-wide and reasonably price. When compared

to other sensing devices, such as glass, paper devices are anywhere from 200 to 1000 times cheaper to produce. An alternative to these instrumental methods is a paper-based analytical device (PAD), which overcomes disadvantages associated with other methods and may allow for inexpensive, direct bedside monitoring of heparin.[1]

Table 1. The properties and impact of a paper based analytical device.[1]

	Property	impact
Mechanical Properties	Flexibility	Low volume of sample required and prevents the breaking of 3D structures if the paper is betn.
	Thickness	
Fibrous/Porous Structure	Soft texture	Delivering exact volume of reagents to the paper, filtration os sample, increase in the number of molecules that can be mobilized
	absorbency	
	Air permeability	
	Network structures	
	High surface-to-volume ratio	
	Capillary action	
Natural origin	Compatible with biological samples	Recyclable, low cost clean up, and thermal stability.
	Easy to sterilize	
	Chemically and biologically inert	
	Disposable and biodegradable	

Paper-based devices are also advantageous because they can an be monitored with a variety of devices, including traditional chemistry instrumentation, such as spectrophotometers and fluorimeters, as well as scanners, cameras, and camera phones. Analysis of the data from

trials using paper-based devices can be analyzed using scanners, cameras, phones, specialized devices (including spectrophotometers or fluorometers), and sophisticated devices such as gel documentation systems. Coupling a PAD with an optical method is the least expensive and the simplest, but they suffer from problems because differences in lighting can cause variations in color intensity and hue. Most methods that deploy PADs are colorimetric and the simplest method for analyzing PAD results is by looking with the naked eye; however this can lead to high variability and problems with reproducibility due to the signal being influenced by the individuals visual perception it is more common to use a camera or smartphone.[1]

PADs have been previously used to analyze small ions, such as sodium and lithium.[20.]Although PADs have been developed for small ion analysis, there are few projects that include work on polyionic species, such as heparin.[3]

Although heparin can be measured with optical and electrochemical methods, these methods have disadvantages, such as the need for expensive instrumentation or sample preparation, and also suffer from interferences by compounding factors.These methods also must be performed in a clinical laboratory rather than a patient's bedside. Further, some third-world countries may not have ready access to such instrumentation. An alternative to these instrumental methods is a paper-based analytical device (PAD). This study adapts traditional optode membrane designs and applies it to a PAD that is then analyzed with a smartphone app.[2,21] Optodes are devices that utilize a polymer membrane containing a color changing material known as a chromoionophore and an ion-exchanger or ionophore. The ionophore selectively interacts with the negatively charged analyte of interest, which causes co-extraction of hydrogen ions into the membrane causing a color change in the chromoionophore as it goes from C to CH^+

(Figure 4). This color change is typically observed using spectroscopic instrumentation; however, this study looks at employing the use of smartphone as a measurement device. In this study, tridodecylmethylammonium (TDMA) is used as the ion-exchanger since it is known to interact with heparin.[2,4] The TDMA ions form a reverse micelle like structure with heparin due to cooperative ion-pairing that occurs between the positively charged TDMA ions and the negative charges of heparin that have a high charge density (Figure 5). This study examines the membrane composition and assay parameters that result in an optimized response to heparin over its clinically relevant range. Specifically, membranes with two different chromoionophores and with or without the polymer poly(vinyl chloride) are examined, as well as response times and the effect of pH to both unfractionated heparin and low molecular weight heparin.

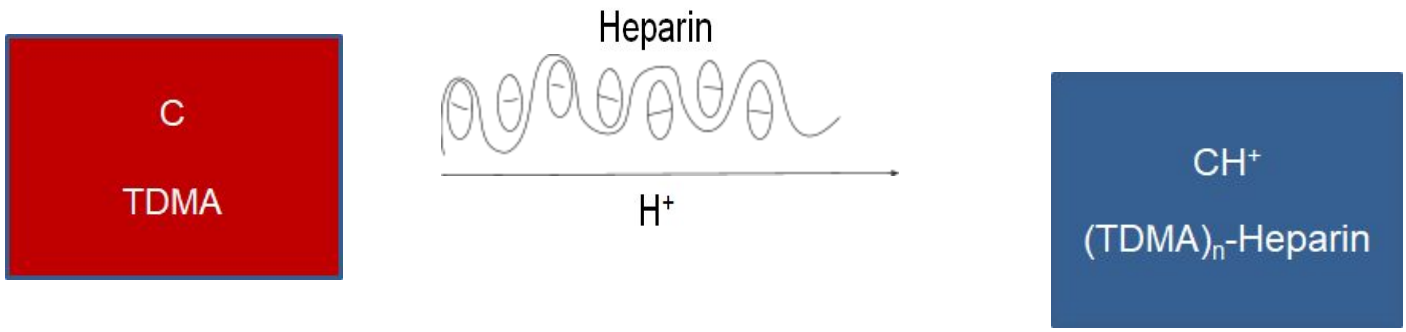

Figure 4. Schematic of the proposed mechanism of the PAD for heparin**.**

Materials and Methods

Development of Paper Based Analytical Device. The membrane was made of tridodecylmethyl ammonium chloride (TDMA), polyvinyl chloride (PVC), chromoionophore ETH 5294,

chromoionophore ETH 2412 and dioctylsebacate (DOS) dissolved in tetrahydrofuran at different weight percentages (Table 2). All chemicals were purchased from Sigma Aldrich (St. Louis, MO). A 2.5 µL aliquot of the membrane solution was pipetted onto F5 filter paper (Fisher Scientific, Hampton, New Hampshire) and dried before an additional 2.5 µL was added. Various concentrations of heparin (Sigma Aldrich) were exposed to each sensing spot and pictures of each were taken after a specified time period. Each experiment was completed in at least duplicate. The photos were analyzed using one of two cell phone apps, either Color Mate ®, Colormeter ®, or Color Assist ®. The background solution of heparin standards varied in pH from 4 to 12. The pH 4.1 buffer was made using a 0.2 M acetate buffer, while the second buffer at pH 7.4 was made using 0.2 M Tris and the pH of 11.7 or 12.12 from 0.20 M phosphate.

Table 2. Membrane compositions evaluated for use with the PAD for heparin.

Membrane	TDMA (wt%)	ETH 5294 (wt%)	DOS (wt%)	PVC (wt %)	ETH 2412
1	1.7	1.0	97.3	0	0
2	2.0	16.0	82.0	0	0
3	5.0	1.0	94.0	0	0
4	1.0	0	97.3	0	1.7
5	1.7	0	97.3	0	1.0
6	1.0	0	77.3	20.0	1.7
7	1.7	1.0	77.3	20.0	0

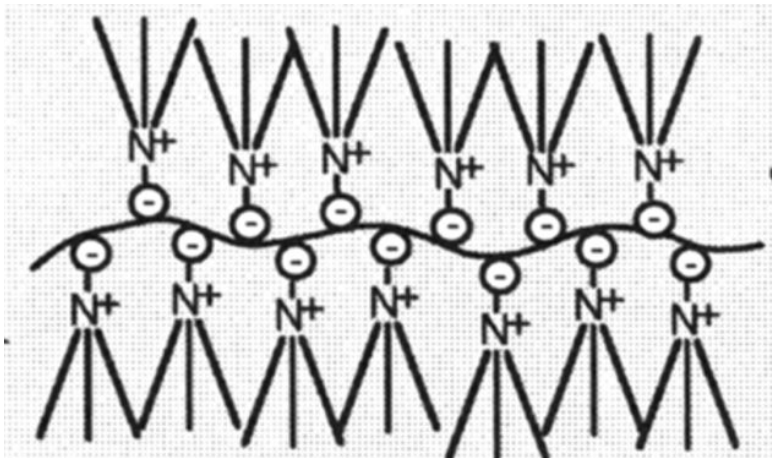

Figure 5. Reverse micelle structure. [21]

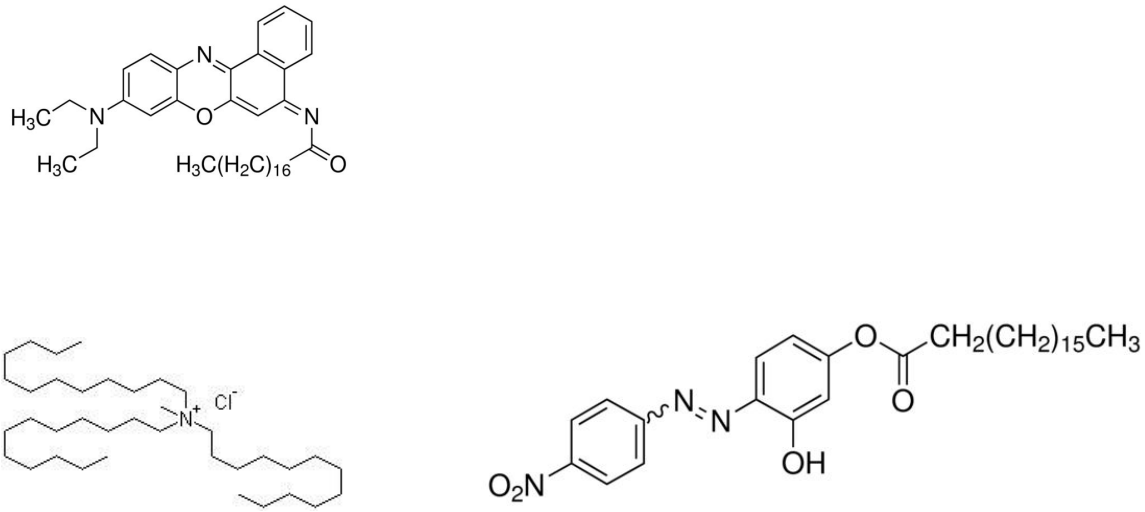

Figure 6. Structures of the ion-exchanger and chromoionophores used in this study. Top left ETH 5294, bottom left TDMA, bottom right ETH 2412.[22,23,24]

Results and Discussion

In order to develop a paper analytical device (PAD) for heparin, several variables were investigated. The effect of PVC, analysis time, membrane composition, volume, and pH of solution were each optimized.

App Analysis

Since the PAD measurement relies on analysis of the color produced by the chromoionophore, reproducible measurements of color are needed. To investigate the best design, pictures of the membrane were taken under different lighting conditions and using different smartphone apps. Originally photos were taken of the membranes' colorimetric response to heparin on a traditional laboratory lighting on the benchtop. However, the difference in lighting due to natural variability caused large relative standard deviations of on average 30-40% and, thus, a lightbox was constructed, which lowered the relative standard deviations to around 3-7%.The box was constructed out of cardboard and contained only one light source, which was a white LED from an LG Aristo phone. There was also a hole through which the inside of the box was observed from the camera lens of a iPod 5s. With initial trials using Color Mate as the measurement tool, the batch-to-batch reproducibility was poor. As a result a trial was run using water and food coloring to determine if the app could distinguish between the different intensities of food coloring, which it could not. This same test was run on different apps, including Color Contrast ®, Colormeter ®, and Color Assist ® and out of all the apps tested Color Assist ® was found to have the best reproducibility having low relative standard deviations of 0.5-7%, which is the range of the relative standard deviation over different concentrations of heparin and different

membrane compositions, in comparison to 30-60% relative standard deviations of the other apps (Figure 7).. Thus, in all future trials Color Assist ® was used.

The Color Assist ® allows for multiple channels to be measured including red, green, blue, hue lightness, and saturation. Of these variables measured lightness and hue were considered to be the most reproducible and were used in future studies. Hue is a feature of monochromatic light that varies with wavelength and is used to denote various regions of the color spectrum and is used to denote adjectives, such as “red”, “yellow”, or “green”. Hue is specifically the dominant wavelength of color and is used to identify the color as distinct from other colors. The lightness is a measurement of how the color of the membrane relates to a scale of white to black and is also used to describe the intensity of the color.

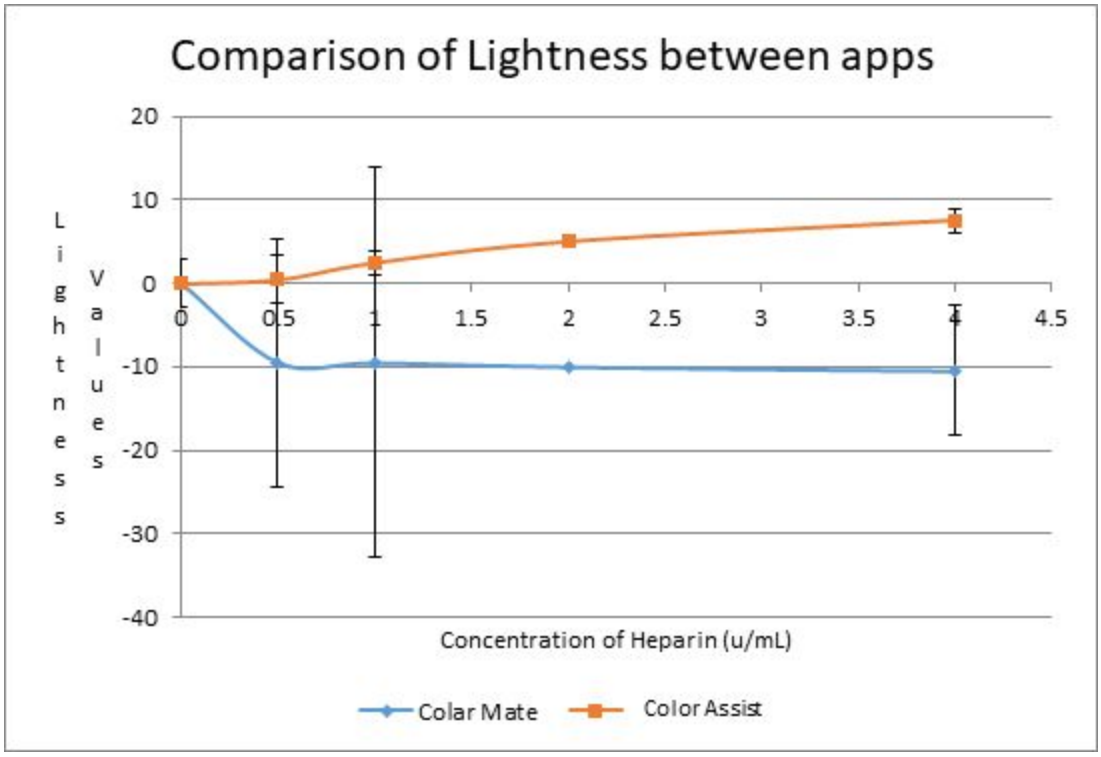

Figure 7. Comparison of cell phone app on heparin signal. Error bars on the graph are a representation of the standard deviations between n number of trials.

ETH 5294

Initial studies were performed using ETH 5294 (Figure 6) as the chromoionophore.

Effect of Polymer on PAD Response

In traditional optodes, polymers are a necessary part of the design to provide a platform for the sensing material. In the PAD, the sensing materials are placed on a membrane filter. Thus, a polymer material might not be necessary. Trials were performed to determine if PVC was essential to the functionality of the membrane. Studies of Membrane 1 (Table 2) with and without traditional polyvinyl chloride (PVC) showed that both have a linear range to 0.6 U/mL of heparin, but the non-PVC sensing material had a greater sensitivity (slope of 26.7 lightness change/U/mL compared to 17.2 for PVC). Thus, PVC was not used in later studies.

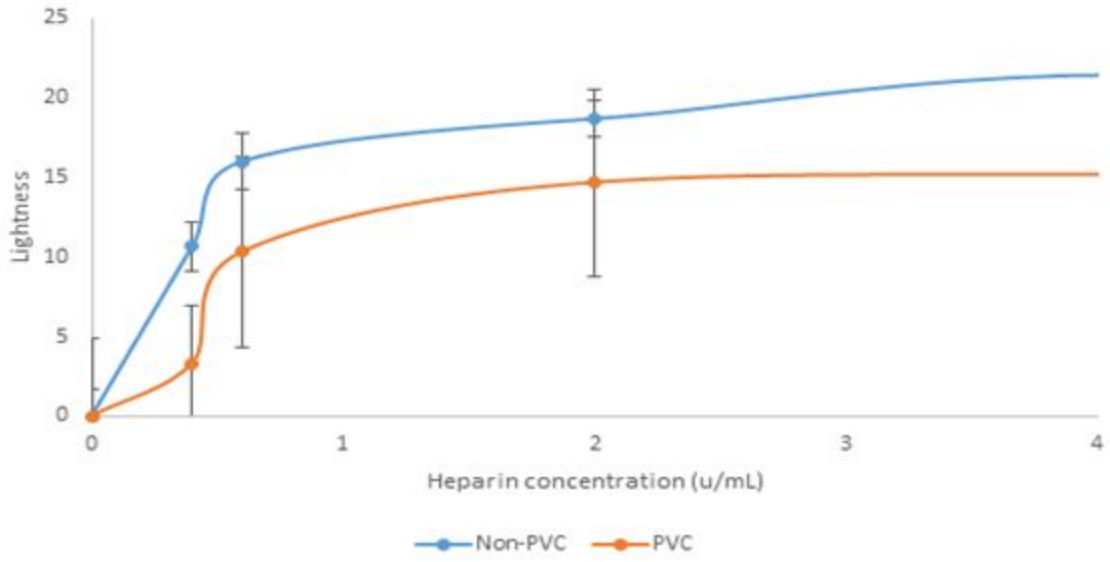

Figure 8. Effect of polymer on lightness values. PVC n=3 and Non-PVC n=4

Effect of Membrane Composition

In order to optimize the response to unfractionated heparin, three different membrane compositions were tested (Table 2). Membrane 1 was found to have the optimal response because it has a larger change is lightness values.The reason for the larger change in lightness values is because there are more molecules of TDMA to complex with heparin meaning there are more hydrogen ions co-extracted, which can then protonate the chromoionophore causing a more noticeable color change. Although it has a lower sensitivity than Membrane 3, it has an extended dynamic range up to 1 U/mL of heparin, which encompasses more of the clinical range. Thus, Membrane 1 was used in remaining studies.

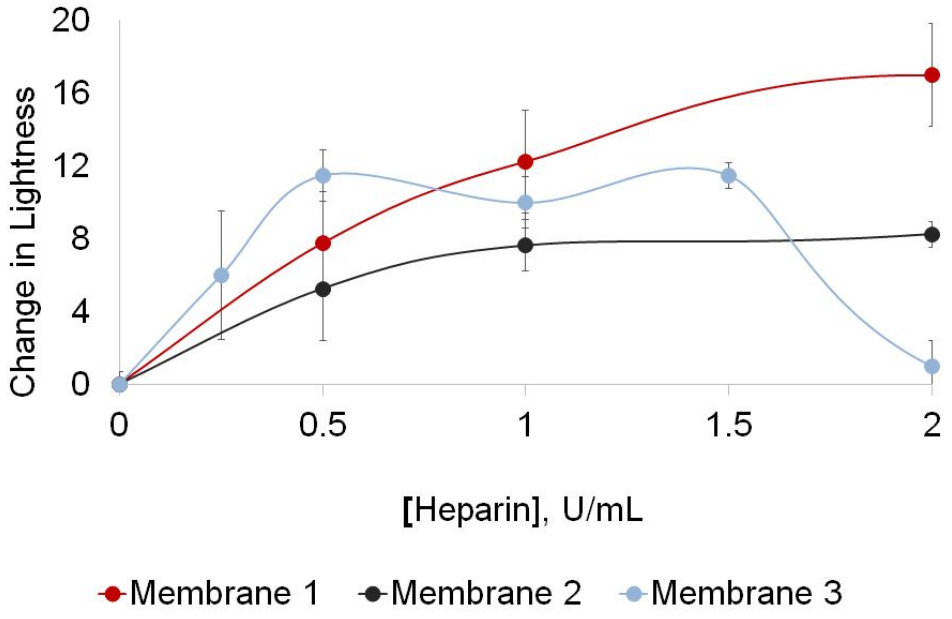

Figure 9. Effect of membrane composition on lightness values. Error bars represent the standard deviation of three trials.

Effect of Time on the Signal

The time of PAD exposure to heparin affected the color development. The limit of linearity increased from 0.5 U/mL at one minute exposure to 2 U/mL after a five minute exposure. No improvement was seen after 10 minutes so five minutes was used for all further studies (data not shown). The initial change that is seen from one to five minutes might be due to the time that it takes for the heparin to the diffuse into the sensing material. Once all the heparin has diffused into the membrane, there will no longer be any more co-extraction of hydrogen ions and thus. why there would be no difference seen in the signals at 10 minutes.

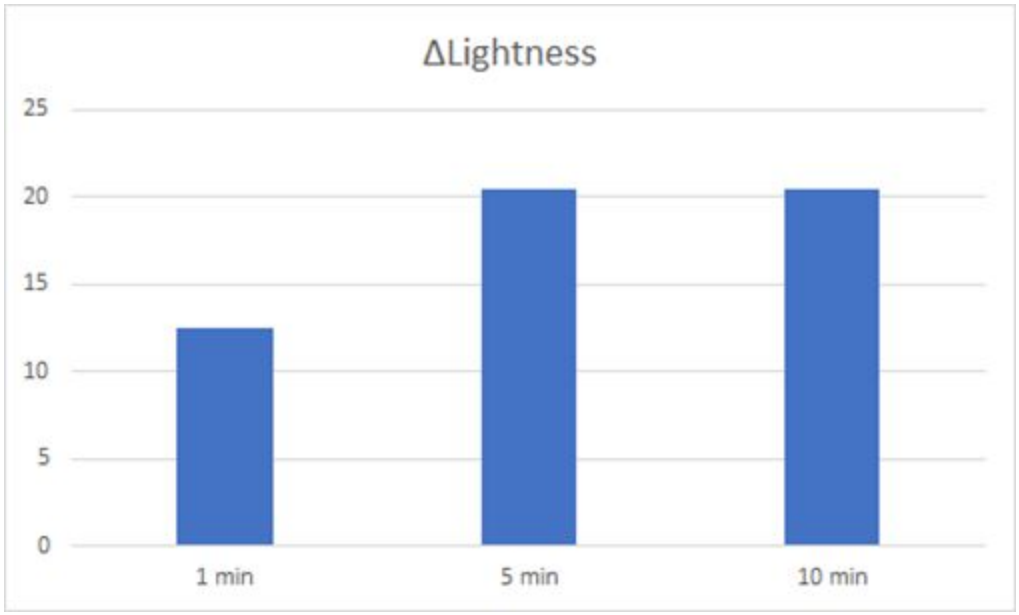

Figure 10. Bar chart of the change in lightness values over their dynamic range.

Effect of Sample Volume on Response

To examine the effect of sample volume on the PAD response, the PAD was exposed to 0.005, 2, and 50 mL of UFH standards. The 2-mL volume had the best sensitivity of 24 change in hue

units/ U/mL compared to the 5-µL which had the lowest sensitivity (Figure 10). However the 5-µL solution had the best dynamic range of 2 U/mL of heparin while the 2-mL solution only had a dynamic range of 0.5 U/mL of heparin. This could be that with a larger volume, there are more moles of heparin present at a particular concentration. Thus, all the ion-exchanger species are interacting with heparin, causing a maximal change in protonation of the chromoionophore. With smaller volumes, a higher concentration is needed to reach the moles needed to interact with all of the ion-exchanger.

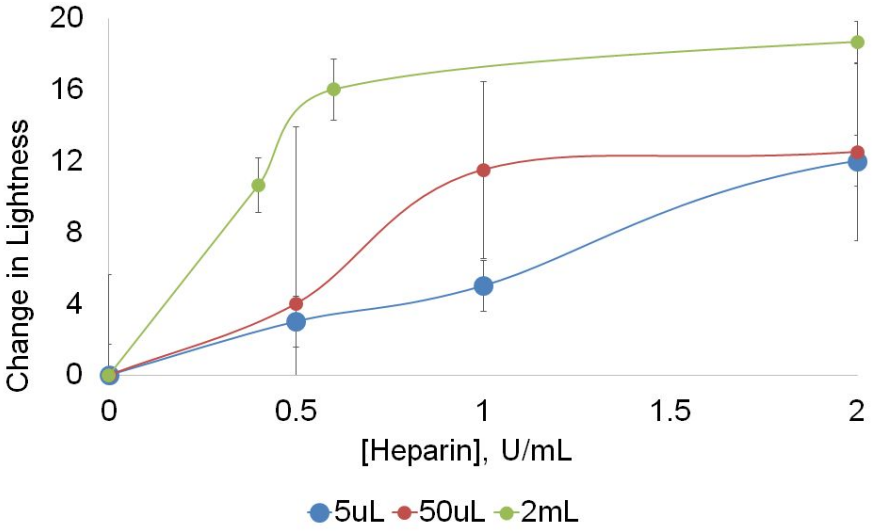

Figure 11. Effect of sample volume on the response of Membrane 1. n=2.

Effect of pH

To determine if pH has an effect on the membrane response, the lightness and hue were measured at a low pH, near neutral pH, and a high pH. As seen in Figure 5, there is no difference

in the lightness values of the membrane in the pH range of 4.1 to 11.7. The pK_a of ETH 5294 is 11.41.[5] Therefore, at a pH of 11.41, half of the chromoionophore would exist in its protonated form, CH+, while half would be in its neutral form, C (Figure 4). At elevated pH, there would be more C compared to CH+. The proposed mechanism of action relies on the colorimetric change that occurs when the chromoionophore is converted from its non-protonated form to its protonated form. Therefore, if a chromoionophore is chosen with a pK_a that is above the pH of blood then their will be less fluctuation in the signal due to changes in the pH of the patients. blood.

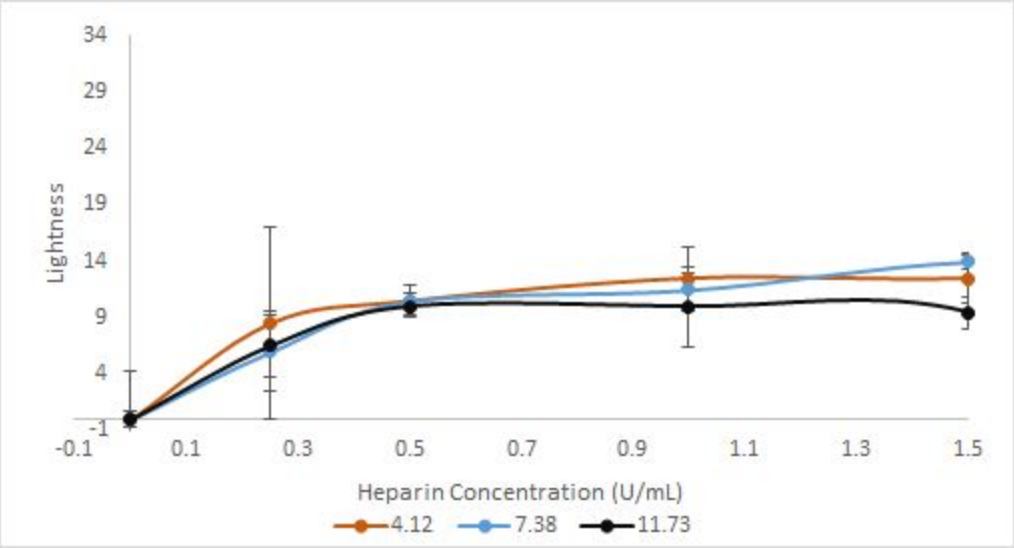

Figure 12. Effect of pH on PAD response to heparin. The lightness at pH values of 4.12, 7.38, and 11.73 are shown. n=3.

ETH 2412

In order to explore more cost effective (80% cheaper) and to investigate the effect of the pK_a of the chromoionophore, ETH 2412 (Figure 6) was used in place of ETH 5294. The pK_a of ETH 2412 is 17.00.[25]

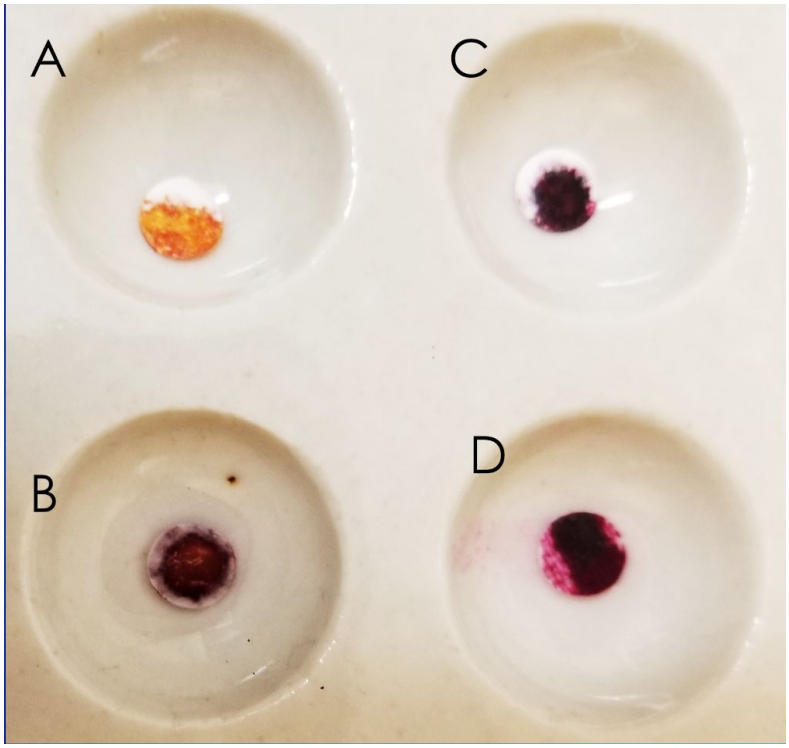

Figure 13. Photos of the sensing material exposed to different heparin concentrations and two different chromoionophores. A. ETH 2412 exposed to 0 U/mL of heparin. B. ETH 2412 exposed to 1 U/mL of heparin. C. ETH 5294 exposed to 0 U/mL of heparin. D. ETH 5294 exposed to 1 U/mL of heparin.

Effect of pH

The pH appears to have no significant effect on the signal of membrane 4 (Figure 13). The reason for this is due to the high pK_a of the ETH 2412 at 17.00.

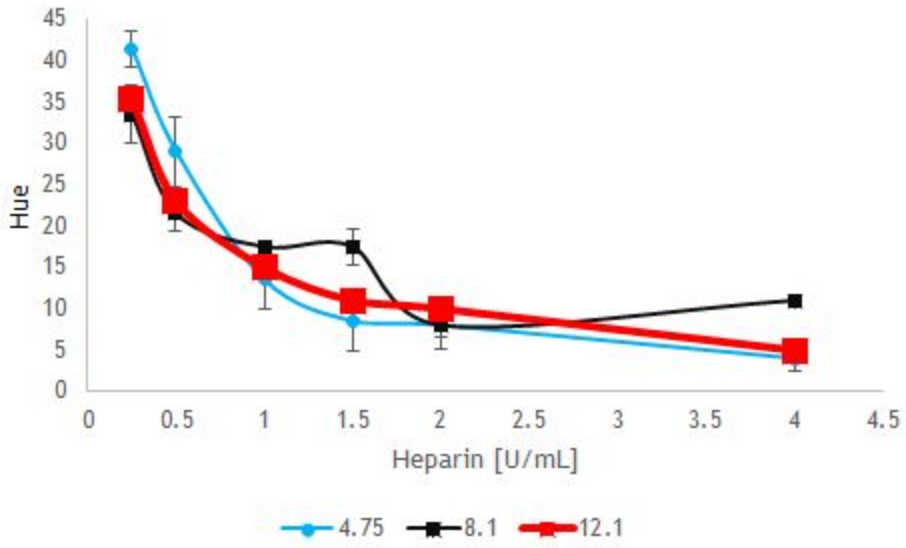

Figure 14. Effect of pH of PAD response on the hue channels. n=4.

Effect of Membrane Composition

When performing trials with ETH 5294, the observed trend was that as the moles of TDMA increased with respect to moles of ETH 5294, the dynamic range increased. In order to determine if the same trend could be applied to ETH 2412 the same analysis was performed. The results (Figure 14) show that increasing the amount of moles of TDMA in comparison to moles of ETH 2412 did increase the dynamic range of the membrane from 0.5 U/mL of heparin to 1 U/mL of heparin as seen in previous trials. This is because as more TDMA binds to heparin, more protons are released and, thus, more protonation of ETH 2412 can occur causing a more colorimetric response.

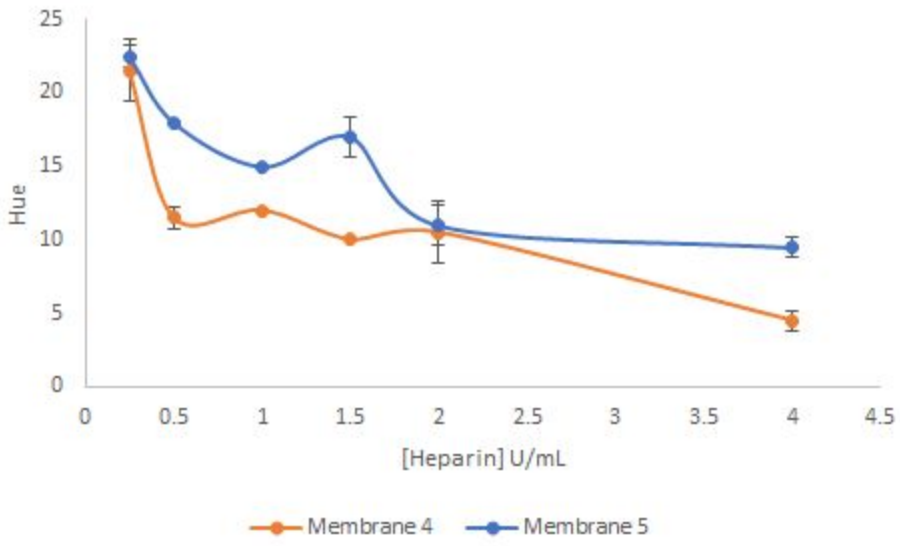

Figure 15. Effect of membrane composition of PAD response on hue channels. n=7 for membrane 4 and n=4 for membrane 4.

Effect of Polymer on PAD Response

When using ETH 5294 PVC was not required as a component in the membrane. After switching to ETH 2412 trials were run to establish if the same conclusions could be drawn. Therefore, trials were performed using membranes 4 and and 6 (Table 2) to determine if PVC did have an effect on the membrane when using ETH 2412. The membrane that included the polymer PVC showed a more linear response and an increased dynamic range up to 2 U/mL heparin (Figure 15).

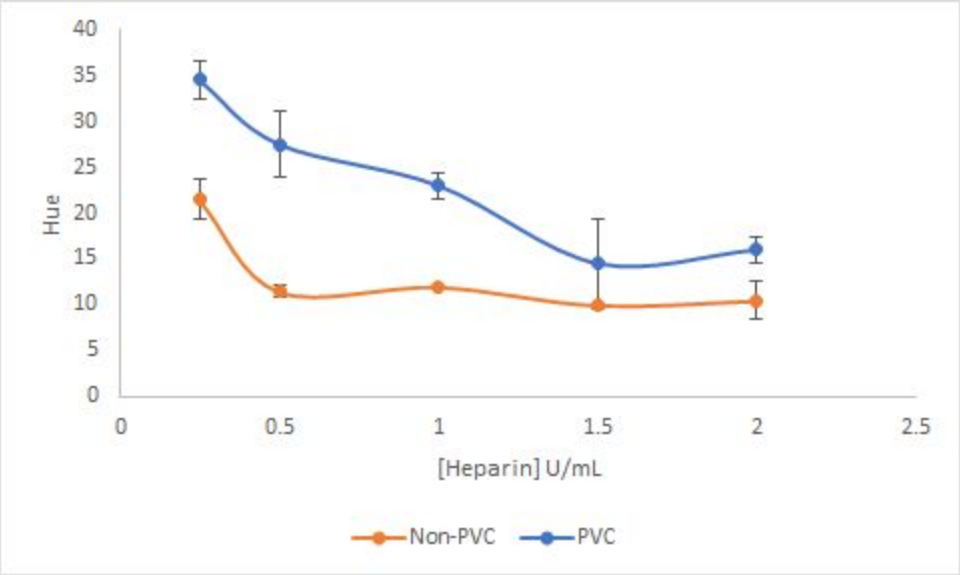

Figure 16. Effect of polymer on the PAD response of lightness channel. n=3 for membrane 6 and n=7 for membrane 4.

Effect of Sample Volume on Response

Trials ran with ETH 5294 showed that the larger volume of 2 mL had the greater sensitivity in comparison with the smaller volume of 5 µL, but the 5 µL solution had the greater dynamic range. This was not the case for ETH 2412 and little change is seen when changing the volume from 2 mL to 50 µL (Figure 16). The 1.5 U/mL reading of the 50 µL sample volume gave an abnormal signal. This could be due to error when pipetting the buffer solution onto the paper device. This trial ideally would be repeated if there was more time to complete the project Therefore, a smaller volume of 5 µL could be used in clinical applications, which is advantageous for the patient.

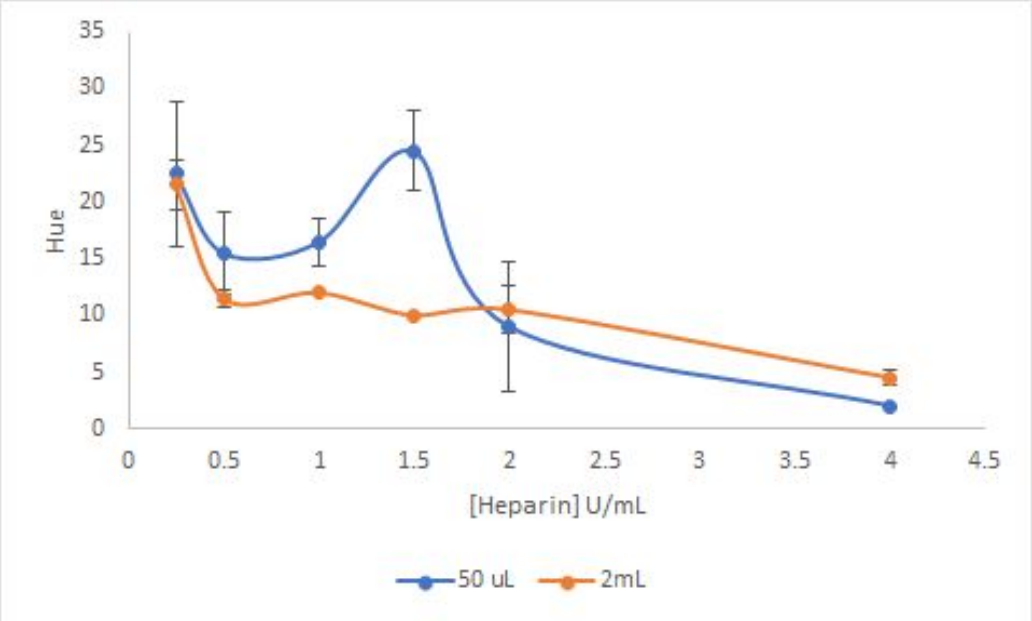

Figure 17. Effect of sample volume on PAD response of hue channel. n=2 for 50 µL and n=7 for 2 mL. The 1.5 U/mL reading of the 50µL sample volume gave an abnormal signal. This could be due to error when pipetting the buffer solution onto the paper device.

Effect of Time on the Signal

There is not a notable difference between the one, five, and ten minute time trials. One minute analysis times could be used, which is advantageous for practical applications.

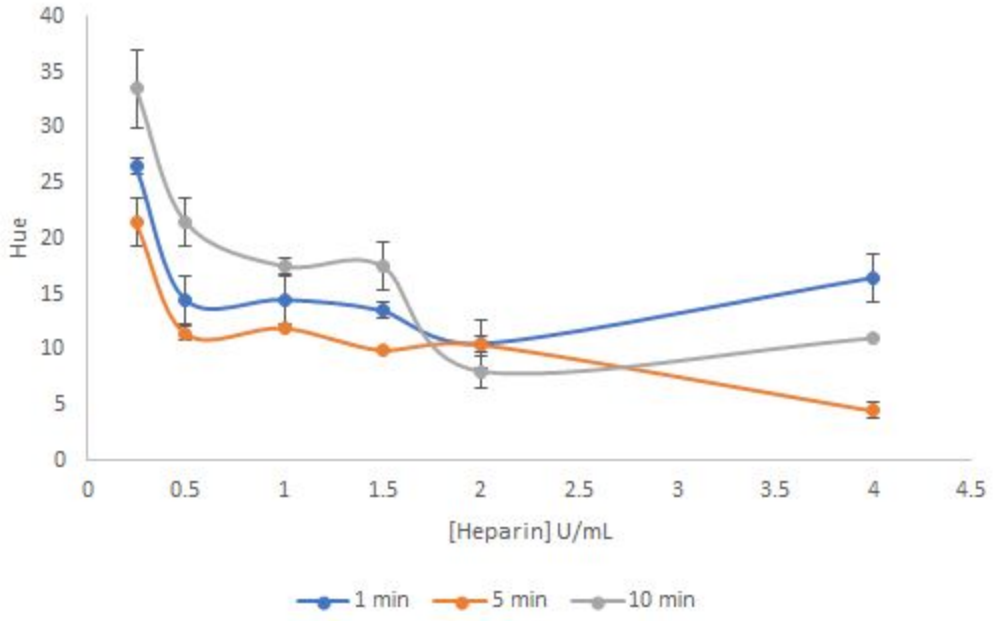

Figure 18. Effect of membrane composition of PAD response on hue channels n=2.

Low Molecular Weight Heparin vs Unfractionated Heparin

In order to determine if the PAD device can measure both LMWH and UFH, which is a limitation of the currently accepted PTT assay that can only measure UFH, tests were ran to analyze the response with increasing concentration of LMWH and UFH. Membrane 4 was also able to quantify LMWH (Figure 18). However, higher concentrations of LWMH were needed to elicit the same response magnitude. This is not surprising since LMWH has fewer negative charges compared to UFH so a greater LWMH molarity is needed to generate the same number of charges and, thus, an equal amount of protonation of the chromoionophore and resulting color change. The dynamic range of the membrane was also extended up to 4 U/mL of heparin with LMWH in comparison to the dynamic range of 2 U/mL of heparin with UFH. The data shown below is specifically the response of membrane 6 to LMWH and UFH; however, trials were run

on membrane 4 as well and the results seen were the same, with LMWH having a longer dynamic range (data not shown).

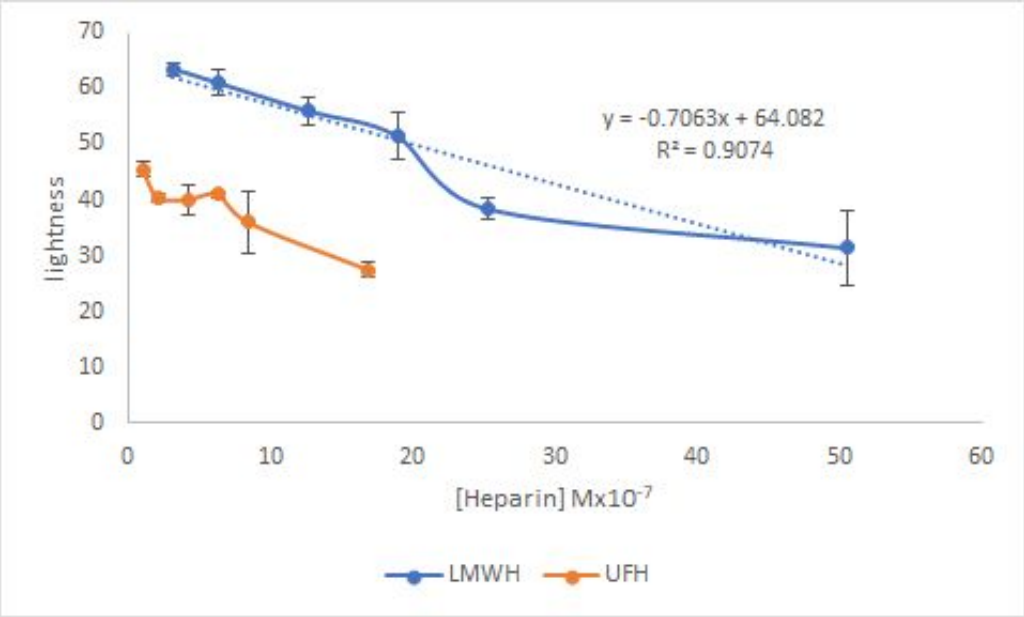

Figure 19. Comparison of membrane 6 response between LMWH and UFH. For LMWH n=2 and UFH n=5.

Plasma Trials

To demonstrate the ability of the PAD to measure heparin in real-world samples, human plasma was spiked with various concentrations of UFH and compared to a calibration curve made from standards in a plasma matrix.

The percent recovery of plasma samples spiked with clinically relevant UFH concentrations was calculated and found to be within acceptable values (Table 3).

Table 3. Percent recovery of plasma samples spiked with UFH.

[UFH], U/mL	Percent Recovery
0.25	99.1
0.50	102
0.75	106
1.0	110

Summary

PADs have seen an increase in popularity in the past 10-20 years due to their low cost, biodegradability, and biocompatibility. This project adds to the analytes for which PADs work, specifically for LMWH and UFH. A PAD for heparin was created with a range of up to 2 U/mL for UFH and 4 U/mL for LMWH. The optimized conditions for the membrane are a 2:1 mole ratio between tridodecylmethyl ammonium chloride (TDMA) and ETH 2412, respectively, and a polymer, such as poly(vinyl) chloride (PVC), a sample volume as small as 5 µL, and an elapsed time of one minute.

Future Direction

More studies are needed to determine an optimal membrane composition for a PAD capable of measuring the full clinical range of heparin, which is up to 8 U/mL. Trials should be run using a chromionophore that has a pKa that is closer to the pH of blood at 7.35 to determine if that will have an effect on the signal of the membrane. Additional studies are needed determine if the

membrane can distinguish between heparin and a contaminant, such as over-sulfated chondroitin sulfate, which was a contaminant that caused an epidemic back in 2008.[26] Other trials should be run on different sulfonation pattern of heparin to determine what effect this will have on the membranes response.

Work Cited

1. Nery, E. W.; Kubota, L. T. *Analytical and Bioanalytical Chemistry* **2013**, *405* (24), 7573–7595.
2. Kim, S. B., Kang, T. Y., Cha, G. S., & Nam, H. (2006), **Quantitative determination of heparin levels in serum with microtiter plate-format optode**. *Analytical Chemistry 557,* 117-122
3. Wang, X.; Mahoney, M.; Meyerhoff, M. E. *Analytical Chemistry* **2017**, *89* (22), 12334–12341.
4. Shu Ching Ma, Victor C. Yang, and Mark E Meyerhoff.(1992), **Heparin-responsive electrochemical sensor: a preliminary study** *Analytical Chemistry 64*, 694-697
5. Wardrop, D.; Keeling, D. *British Journal of Haematology* **2008**, *141* (6), 757–763.
6. **"Heparin (Heparin): Side Effects, Interactions, Warning, Dosage & Uses."** RxList. N.p., n.d. Web. 13 Nov. 2016**.**
7. **"Coagulation Cascade."** Coagedex. N.p., May 2016. Web. Nov. 2016.
8. Guerrini, M., Guglieri, S., Beccati, D., Torri, G., Viskov, C., & Mourier, P. (2006). **Conformational transitions induced in heparin octasaccharides by binding with antithrombin III**. *Biochemical Journal, 399*(2), 191-198. doi:10.1042/bj20060656
9. Gormsen, J., & Kruse-Blinkenberg, H. O. (2009). **Heparin And Its Clinical Use**. *Scandinavian Journal of Haematology, 23*(S34), 52-60. doi:10.1111/j.1600-0609.1979.tb01574.x

10. Tiuca, I. (2016). **Biochemistry and Stereochemistry of Anticoagulants**. *Anticoagulation Therapy*. doi:10.5772/64417

11. Hirsh, J., & Fuster, V. (1994). **Guide to anticoagulant therapy. Part 1: Heparin**. American Heart Association. *Circulation, 89*(3), 1449-1468. doi:10.1161/01.cir.89.3.1449

12. Ma, S. C.; Yang, V. C.; Meyerhoff, M. E. *Analytical Chemistry* **1992**, *64* (6), 694–697.

13. Bounameaux, H. *Vascular Medicine* **1998**, *3* (1), 41–46.

14. Basu,Dilip; Gallus,Alexander; Hirsh,Jack; Cade,John (1972), **A Prospective Study of the Value of Monitoring Heparin Treatment with the Activated Partial Thromboplastin Time***, N.Engl.J.Med*., 287, 324-327

15. Hirsh, J., Anand, S. S., Halperin, J. L., & Fuster, V. (2001). **Mechanism of Action and Pharmacology of Unfractionated Heparin**. *Arteriosclerosis, Thrombosis, and Vascular Biology, 21*(7), 1094-1096. doi:10.1161/hq0701.093686

16. Jairajpuri, M., Rashid, Q., & Abid, M. (2014). **Elucidating the specificity of non-heparin-based conformational activators of antithrombin for factor Xa inhibition.** *Journal of Natural Science, Biology and Medicine, 5*(1), 36. doi:10.4103/0976-9668.127282

17. Lammers, G., Westerlo, E. M., Versteeg, E. M., Kuppevelt, T. H., & Daamen, W. F. (2011). **A Comparison of Seven Methods to Analyze Heparin in Biomaterials:**

18. Gemene, K. L.; Bakker, E. *Analytical Biochemistry* **2009**, *386* (2), 276–281.

19. Xuewei Yang, Yu Qin, Mark E. Meyerhoff (2015) **Paper-based plasticizer-free sodium ion-selective sensor with camera phone as a detector** *Chem.Commun 51*, 15176-15179

20. Lammers, G., Westerlo, E. M., Versteeg, E. M., Kuppevelt, T. H., & Daamen, W. F. (2011). **A Comparison of Seven Methods to Analyze Heparin in Biomaterials: Quantification, Location, and Anticoagulant Activity**. *Tissue Engineering Part C: Methods, 17*(6), 669-676. doi:10.1089/ten.tec.2011.0010

21. Fu, B.; Bakker, E.; Yang, V. C.; Meyerhoff, M. E. *Macromolecules* **1995**, *28* (17), 5834–5840.

22. **"(TDMAC, N,n-didodecyl-n-methyl-1-dodecanaminiuchloride, 7173-54-8, Arquad)Tridodecyl Methyl Ammonium Chloride."** , View Tridodecyl Methyl Ammonium Chloride, XMPT Product Details from Anhui Benma Pioneer Technology Co., Ltd. on Alibaba.com. N.p., n.d. Web. 13 Nov. 2016.

23. Chromoionophore I (ETH 5294) 27086 https://www.sigmaaldrich.com/catalog/product/sial/27086?lang=en®ion=US (accessed Feb 21, 2018).

24. Chromoionophore IV(ETH 2412) 27089 https://www.sigmaaldrich.com/catalog/product/sial/27089?lang=en®ion=US (accessed Feb 21, 2018).

25. Qin, Y.; Bakker, E. *Talanta* **2002**, *58* (5), 909–918.

26. Guerrini, Marco, et al. "**Oversulfated Chondroitin Sulfate Is a Contaminant in Heparin Associated with Adverse Clinical Events**." *Nature Biotechnology*, vol. 26, no. 6, 2008, pp. 669–675., doi:10.1038/nbt1407.