Point-of-care Sensor for Creatinine Detection

by

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ABSTRACT

Cardiovascular disease is affecting millions of people worldwide and is the leading cause of death in the United States. This disease is closely related to the abnormal creatinine levels in blood. For this reason, there is a need for a low-cost point-of-care device that could measure the creatinine level in blood with the goal of managing and preventing cardiovascular disease. This project introduces a Molecular Reactive Lateral Flow Assay (MoReLFA) device that is aimed toward creatinine detection based on an optimized chemical reaction of creatinine and alkaline picrate. The device consists of different membranes that accommodate 50 microliters of fluid sample and carry out a colorimetric reaction, in which deposited-colored region is analyzed for Red, Green, and Blue (RGB) components via an image processing software. The color intensity from the RGB outputs was then studied and compared with a gold standard spectrophotometry-based technique. The results show that the MoReLFA sensor could successfully detect creatinine levels in standard solutions. The plot of the sensor color intensity against the absorbance from spectrophotometry shows a good correlation between the two methods (R^2 = 0.96). Furthermore, the paper introduces the development of a RGB reader box that is portable and for easy assessment of RGB values. The color intensity from the box shows an increasing trend with increasing creatinine concentrations; and the coefficient of determination of this relationship is 0.85.

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CHAPTER 1

INTRODUCTION

1.1 Research Motivation

According to CDC, cardiovascular disease is the leading cause of death in the United States[1]. For a patient to successfully manage cardiovascular disease, dynamic blood creatinine and potassium (K+) levels must be monitored[2,3,4,5]. Unfortunately, the current means to monitor K+ and creatinine outside hospitals and clinics do not exist. For this reason, there is a need for a point-of-care (PoC) device to cover existing gaps for in-home personalized treatment.

1.2 Innovation of Lateral Flow Analysis for Creatinine Detection

Lateral flow analysis (LFA) based on immunochemical reactions has played a vital role in the current pandemic times. Rapid SARS-CoV-2 tests or SARS-CoV-2 antibodies are currently tools to regulate decisions on infection and vaccination levels[6]. The main reason for these LFA tests' success is the high analytical accuracy, user-friendly operation, inexpensive cost, and instant naked-eye result reading[7]. The tests serve as an alternative to lab-based immunological tests and are based on principles such as pregnancy tests and other peptides and proteins tests to detect high molecular weight molecules[8]. However, besides the plethora of work done in LFA for biological fluids, there is a lack of LFA devices for detecting low molecular weight molecules due to fundamental challenges posed by this type of molecules' detection[9]. This problem significantly impacts the ability to detect many analytes crucial to sustaining homeostasis of vital organs' physiology, such as

the heart and the kidney. Currently, LFA devices are not immunologically based, use enzymes as sensing response amplifiers for detecting low molecular weight molecules. The problem with the use of enzymes is that enzymes face denaturation challenges and stability, requiring low-temperature storage of LFA devices and making it difficult for LFA's application in real scenarios[10,11]. If the low molecular weight analytes were detected through LFA without the need for enzymes or antibodies, it would be possible to achieve accurate analysis without special storage conditions. In clinical chemistry, analyte-selective chemical reactions have been used to detect low molecular weight analytes, but relying on sample pretreatments of centrifugation or chemical "digestion" in laboratory[12]. For these reasons, the aim of this project is to integrate analyte-selective chemical reactions and sample pretreatments to LFA, reducing the constraints and enabling adoption for timely and proper applications for creatinine detection.

CHAPTER 2

MORELFA DEVICE FOR CREATININE DETECTION

To eliminate the use of antibodies or enzymes and overcome the challenges of detecting low molecular weight molecules, a device called Molecular Reactive Lateral Flow Analysis (MoReLFA) is introduced in this paper. A MoReLFA device is configured with three different components as shown in Figure 1, including: 1- analyte extraction mechanism from complex matrix samples, 2- selective chemical reaction, and 3- sensitive detection. All together not only allows for miniaturization but also ensures selectivity and use in real complex samples without previous treatment.

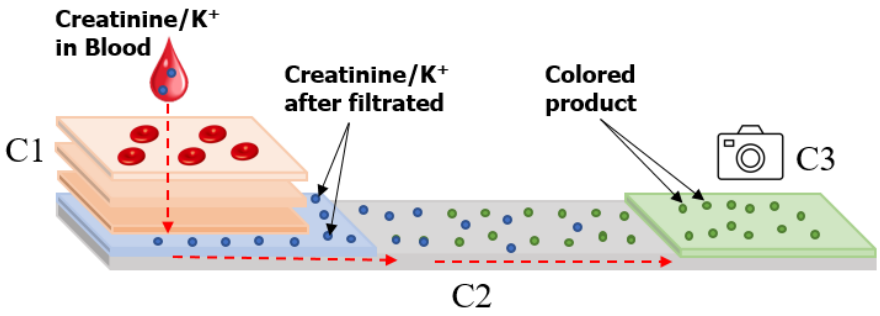

Figure 1: *Schematic Representation of More Lateral Flow Analysis Device with Component 1, 2 and 3 (C1, C2, and C3)*

The first component, analyte extraction mechanism, consists of fiber blend material (blood separator) and a conjugate pad. The blood separator will trap the unwanted white cells (12-17 μm), red cells (7-8 μm), and platelets (2-4 μm)[13], and leaving the plasma as the sole liquid matrix portion of the sample for analysis; a conjugate pad which receives

the plasma provides reagent that involves in the reaction with the analyte. In addition, the analyte extraction mechanism also relies on a porous channel that will be designed to further separate low molecular weight and soluble analytes in the plasma. The porous channel is modified with a nitrocellulose matrix to promote the adsorption of hydrophobic elements such as albumin, other proteins, and lipids in the plasma's flow head. This will further help to eliminate interferences for component 2. A selective chemical reaction will be achieved in component 2 with specific reagents that can selectively react with creatinine as an analyte. The reaction is a chemical reaction based on covalent bonds formation for detection of creatinine. The reagents are then deposited downstream on the wick pad in component 3, where significant creatinine purification can be achieved. The reaction produces specific-colored products, which can be sensitively detected by capturing the specific-colored products. The different concentrations of the final products will generate color development that can be read. This entire approach will create a molecular reactive flow, which gives name to the method.

CHAPTER 3

METHODS

3.1 Study Protocol

A certified laboratory method for creatinine quantification in human serum was adopted in this study. The protocol uses a Beckman Coulter UniCel® DxC 800 Synchron Clinical System to determine creatinine concentration using Jaffe rate method[14]. The Jaffe rate method involves the reaction of picric acid with creatinine in alkaline condition and produce an orange-red complex product[15]. The reaction mechanism of the Jaffe method is shown in figure 2 below:

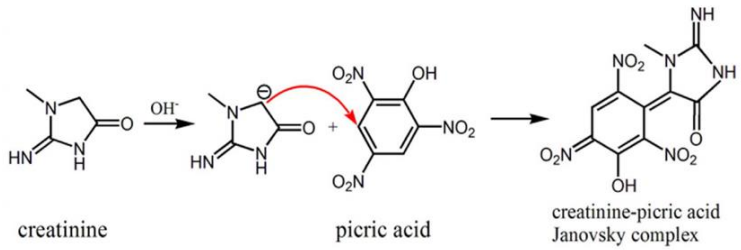

Figure 2: *Reaction of Creatinine with Alkaline Picric Acid to Produce a Colored Complex*

The reagents for this method include 0.05 M picric acid and 0.188 M sodium hydroxide mixed together with volume ratio of 4:1. The total test volume on the instrument is 570 µL including 16.5 µL sample and 553.5 µL of reagents mixture. The absorbance is obtained at 520 nm between 10 and 25 seconds after sample injection. The absorbance has been shown to be directly proportional to the creatinine concentration at 520 nm[16,17,18].

3.2 Modified Method Adapting to the Use of Available Spectrophotometer

Due to the laboratory settings, the study used the SpectraMax™ M5 instead of Synchron Clinical System to obtain the absorbance data after 5 minutes. The overall concentration of picric acid and sodium hydroxide in each reaction well was kept the same, but the volume of the creatinine standard was scaled up to 129.2 µL in a total reaction volume of 200 µL for more sensitivity in the measurements.

3.3 Strategy for Creatinine Quantification on a Lateral Flow Strip

The assembling of a MoReLFA device follows the steps in figure 3, these steps include: 1- sticking the gloss film side of the nitrocellulose membrane onto the backing card with picric acid previously casted, 2- followed by the wick pad on the top end of the backing card, 3- then followed by the conjugate pad that was previously casted with sodium hydroxide, and 4- lastly the sample pad at the other end of the backing card. The volume of picric acid 1% and sodium hydroxide 1M used were 11.13 µL and 7.33 µL to accommodate 50 µL of sample solution. Figure 4 shows the completed assembled MoReLFA device with proposed components.

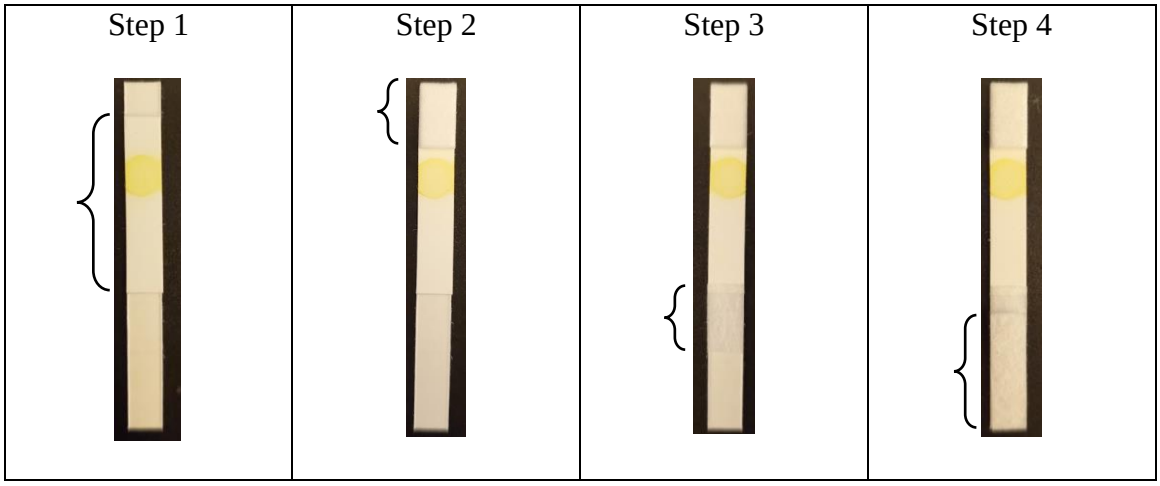

Figure 3: *Assembling Steps of the MoReLFA Device*

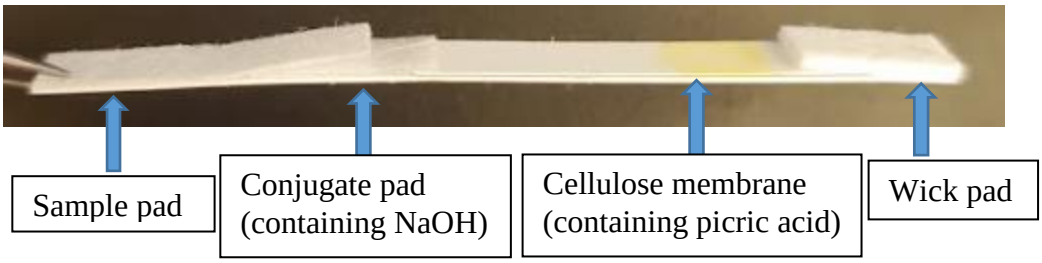

Figure 4: *Assembled MoReLFA Device*

As described in the design section of the lateral flow device, the reagents and the colored product of the creatinine/alkaline picrate reaction are deposited on the wick pad in component 3 of the strip. The developed color on this wick pad will be captured and analyzed for creatinine quantification. The red (R), green (G), and blue (B) components of the color shown on the wick pad were obtained using ImageJ software and the absorbance is found by the following equation, where I is the intensity rendered from RGB component:

$$Absolute\ absorbance = -\log\left(\frac{I_{sensing}}{I_{reference}}\right) \quad (1)$$

The intensity shown in equation (2) for each wick pad was also used for creatinine quantification:

$$Intensity = \left|\left(\frac{I_{sample}}{I_{blank}}\right) - 1\right| \quad (2)$$

3.4 Materials

Four different membranes were used in the MoReLFA sensor design: a sample pad of grade 1662 fiber-blend material (0.61 mm thickness) purchased from Ahlstrom™, a conjugate release pad grade 8950 from Ahlstrom™, a nitrocellulose membrane (FF170HP) with thickness of 200 µm from Whatman™, and a wicking or absorbent pad from Ahlstrom™. These four membranes are fixed together with an adhesive vinyl backing card.

The test strip was design to have a dimension of 57 by 5 mm. All the pads were cut with the same width of 5 mm. The sample pads, wick pads, conjugate pads, and the nitrocellulose membranes were cut with length of 18 mm, 9mm, 10mm, and 25mm, respectively. These membranes and the backing card were cut using the Cricut Maker cutter. Spectrophotometric measurements were obtained from SpectraMax™ M5 set on absorbance mode and endpoint analysis at 520 nm. The calibration curve was obtained for creatinine concentrations from 0 to 200 µM for every 10 µM increment. Creatinine standards were prepared from its anhydrous form (Sigma-Aldrich, ≥ 98% purity). Reagents for chemical reaction with creatinine include picric acid (Sigma-Aldrich, 0.9 – 1.1%), and anhydrous sodium hydroxide (Sigma-Aldrich, ≥ 98% purity). Lithium-heparin tubes were used to collect venous blood draws.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Absorbance of Creatinine Standards from Spectrophotometer

Figure 5 shows the calibration curve for the absorbance of different creatinine concentrations from 0 to 200 µM after 5 minutes of incubation time. The error bars were included for 3 replicates to account for the variability and consistency in the measurements. The calibration curve shows a linear trend with coefficient of determination of 0.997 indicating there is very minimal deviation of the data points from the calibration curve.

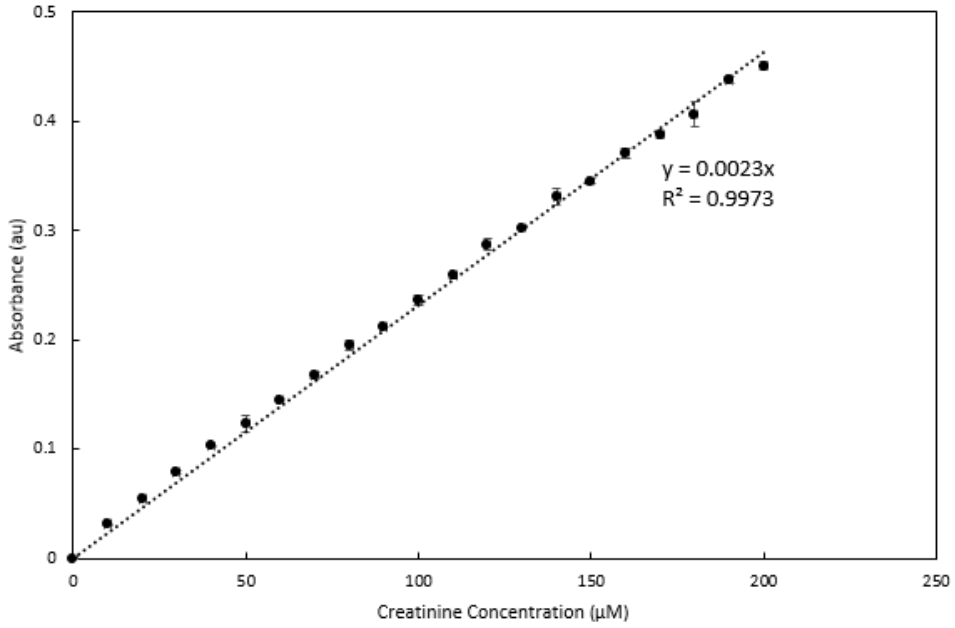

Figure 5: *Absorbance of Creatinine for Different Concentrations*

The experiment was also repeated for the absorbance of different creatinine concentration as a function of time. As seen in Figure 6, there is negligible changes in the absorbance measurements versus time for each creatinine concentration point.

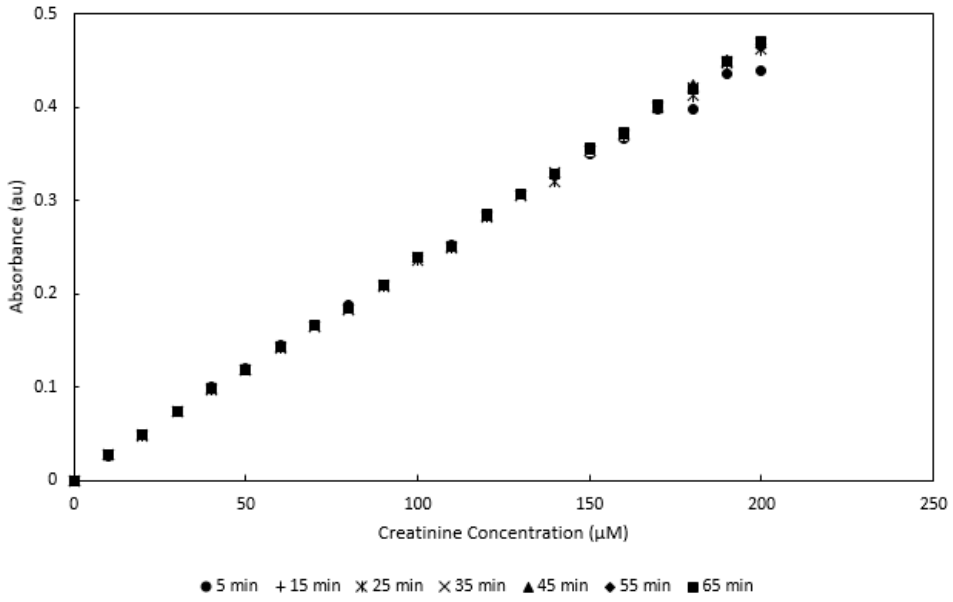

Figure 6: *Absorbance of Creatinine at Different Time*

4.2 Tested Ratio of Alkaline Picrate Solution

As proposed in the protocol, the overall concentrations of picric acid and sodium hydroxide in each reaction well are 0.00972 M and 0.147 M, respectively. This original concentration ratio of picric acid and sodium hydroxide was set at 1[PA]:1[N], in which 1[PA] corresponds to picric acid concentration of 0.00972 M and 1[N] corresponds to sodium concentration of 0.147 M. In order to confirm the effectiveness of this reagent mixture, different concentrations ratio of picric acid and sodium hydroxide were tested.

As seen in figure 7a, in the case of keeping the concentration of sodium hydroxide the same while increasing the concentration of picric acid in 2, 3, and 4 times, the original ratio of 1[PA]:1[N] shows the most distinction in the absorbance of 50, 90, and 120 µM creatinine. Figure 7b, the case of keeping concentration of picric acid the same while increasing the concentration of sodium hydroxide, shows very small distinction for the

absorbance of different creatinine concentration, especially for high concentration of sodium hydroxide. As a result, the original concentration ratio of the two reagents was kept the same and carried forward for further experimental studies.

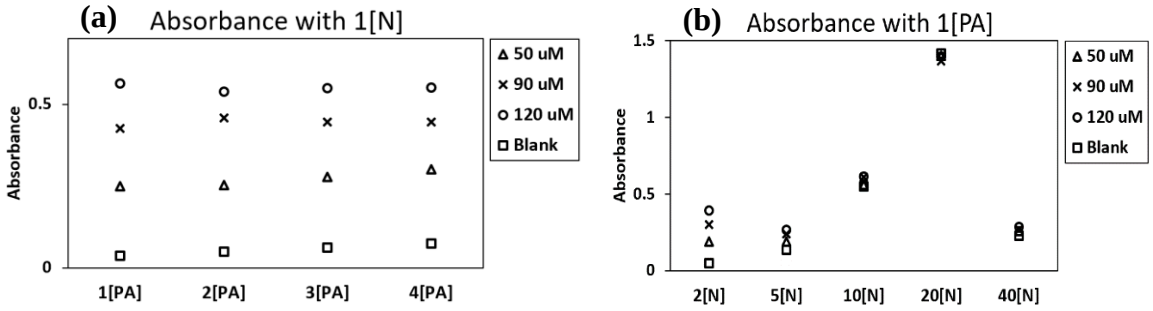

Figure 7: *Tested Ratio of Picric Acid and Sodium Hydroxide for (a) Different Concentrations of Picric Acid and (b) Different Concentrations of Sodium Hydroxide*

4.3 Tuning Conditions on Solid Substrates

Before testing creatinine on the actual MoReLFA device, it was first tested on a single piece of wick pad to observe the chemistry and color change in the solid phase. Creatinine was mixed well with alkaline picrate solution and the creatinine/alkaline picrate mixture was added onto an 8 by 8mm wick pad with another pure white wick pad on top to serve as a reference area. Picture after 5 minutes of adding the solution (Figure 8) was captured and used with ImageJ software for RGB color analysis.

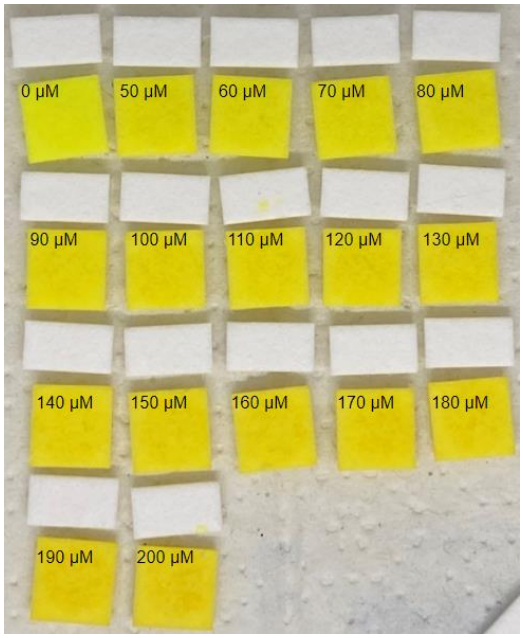

Figure 8: *Picture of Different Creatinine Concentrations in Alkaline Picrate Solution after 5 Minutes of Adding onto a Single Wick Pad*

Figure 9 shows the plot for the absorbance of the green component for different creatinine concentrations on each wick pad. Even though a few data points such as at 60, 170, and 180 µM are little off from the trend, the overall linear fit looks very good with a coefficient of determination of 0.97.

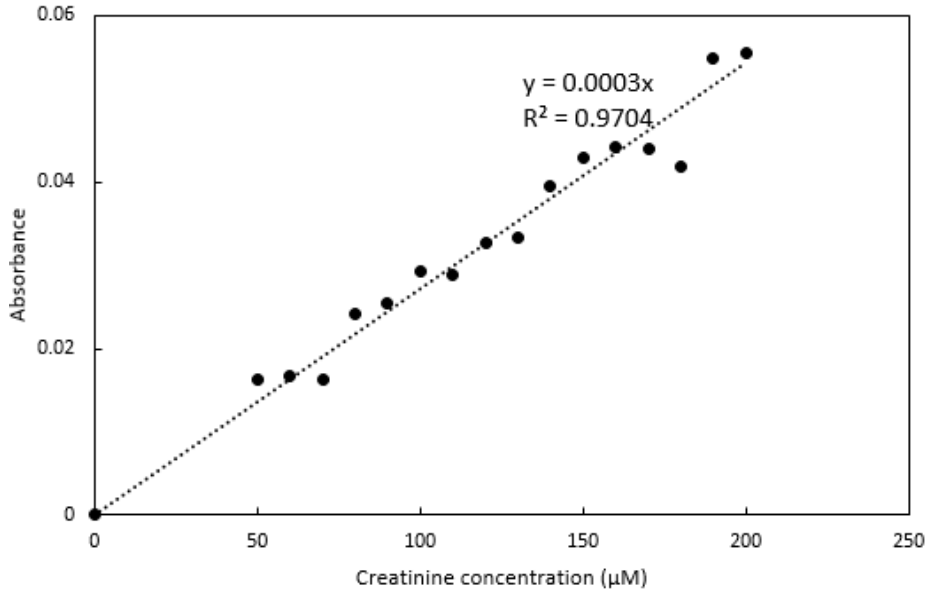

Figure 9: *Absorbance of Creatinine/Alkaline Picrate on Wick Pad*

Based on the absorbance obtained from each wick pad of different creatinine concentrations, this absorbance is used to compared to the one obtained from spectrophotometry. This is done by plotting the absorbance from the wick pad against the absorbance from spectrophotometry as seen in figure 10. The fitted equation indicates that the absorbance from the wick pad is well correlated to the absorbance from spectrophotometry.

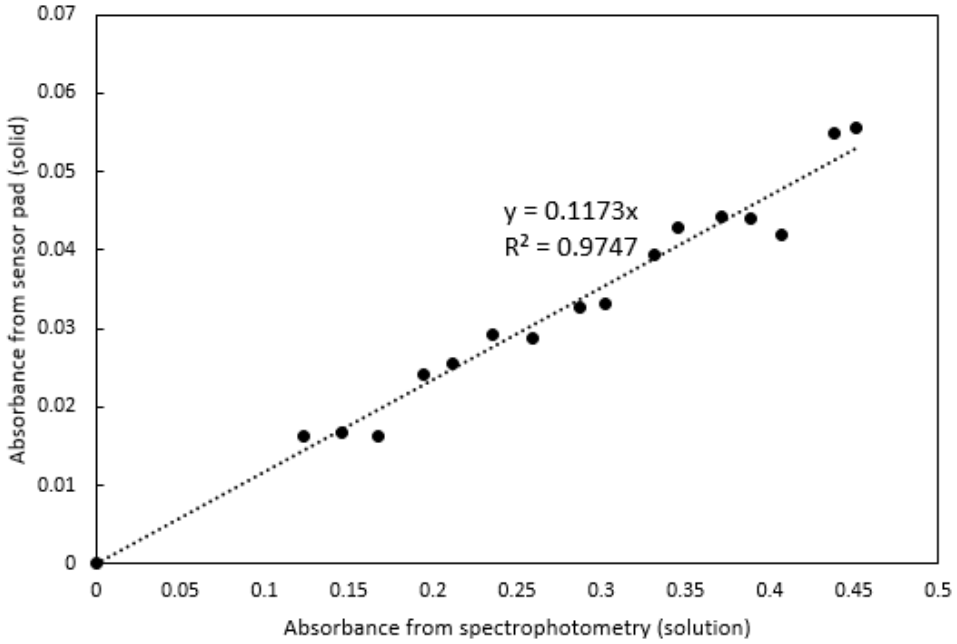

Figure 10: *Correlation Plot Between Wick Pad Outputs Versus Spectrophotometer Outputs*

From the previous results in figure 9 and 10, the chemistry on the solid phase was perceived to have a great potential when moving to the proposed MoReLFA device. It was observed that with 50 µL, there was not enough sample volume to wet the wick pad uniformly; therefore, the wick pad was replaced with the sample pad with the same dimension for the deposited reagent. Figure 11 is the picture of the tested MoReLFA strips taken after 10 minutes of adding the standards with creatinine concentrations of 0, 50, 70, 90, 120, and 200 µM.

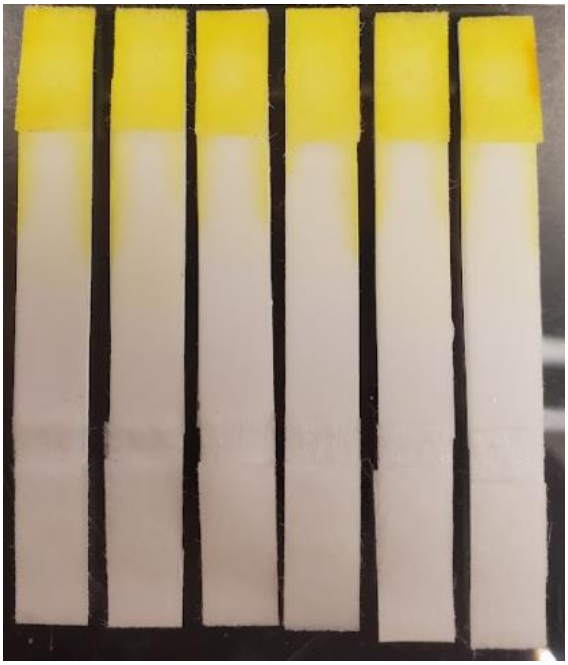

Figure 11: *Tested MoReLFA Strips after 10 Minutes of Running Time. Standard Creatinine Concentrations from Left to Right Are 0, 50, 70, 90, 120, 20 µM, Respectively*

Figure 12 shown below is the relationship between the intensity obtained from the above picture and different creatinine concentrations, and figure 13 is the correlation plot between the results obtained from the color intensity of the sensor and the absorbance from spectrophotometer. Even though it is difficult to detect the difference in the color intensity of each sensor with naked eye, the plot is the increasing linear trend for increasing concentrations. The fit was considered to be quite good with the coefficient of determination of 0.96 for both intensity and correlation plots.

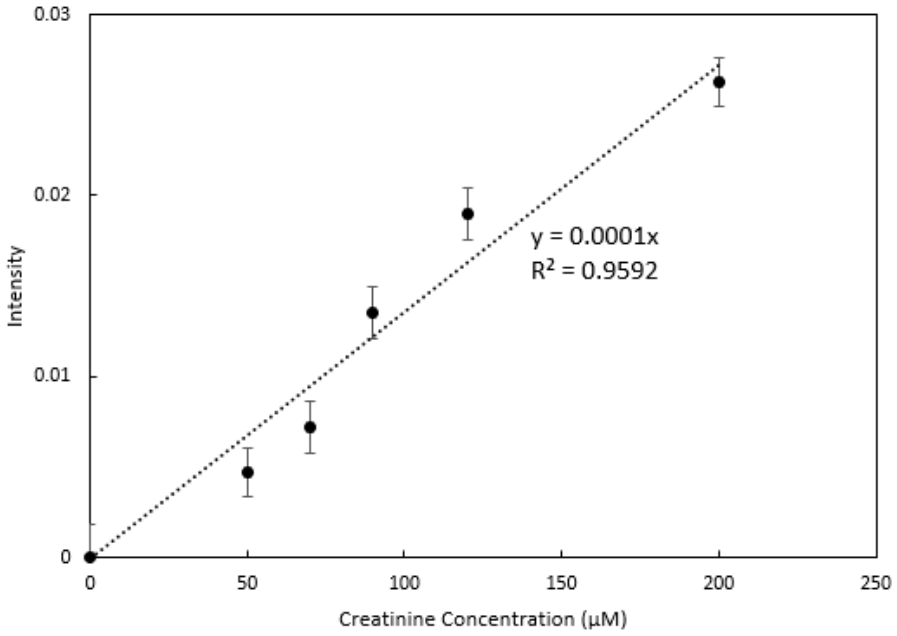

Figure 12: *Relationship Between Sensor Color Intensity and Creatinine Concentrations*

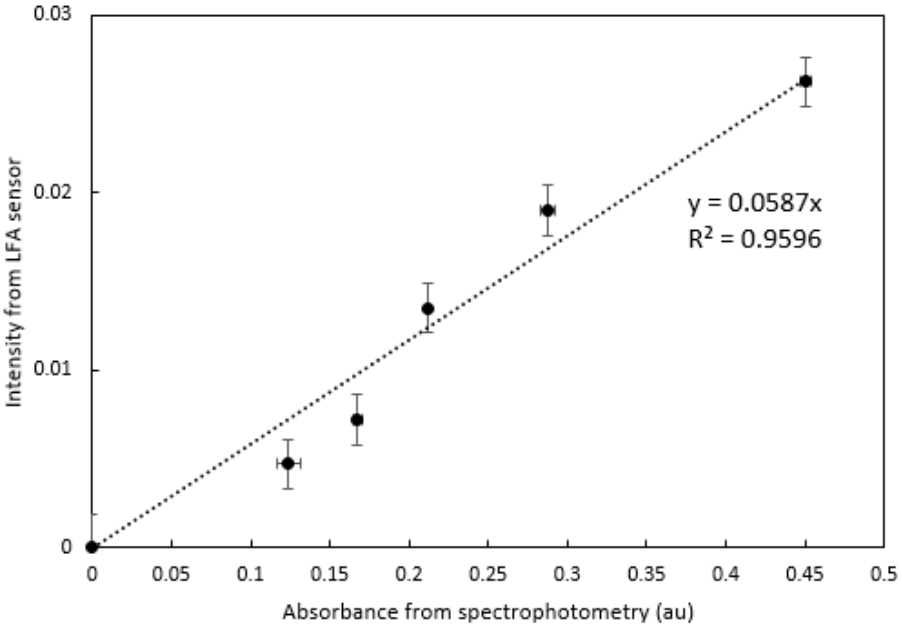

Figure 13: *Correlation Plot Between Sensor Color Intensity and Spectrophotometric Absorbance*

4.4 Absorbance of Plasma from Spectrophotometer

With the proposed method, three healthy human subjects (ages: 22 – 52, 2 males and 1 female) were chosen for three venous blood draws. The blood samples were collected in lithium heparin tubes and were centrifuge with the speed of 3500 rpm for 10 minutes to obtain the plasma containing creatinine analyte. The plasma of each subject was then added to the alkaline picrate solution in the 96-well plate and run in the spectrophotometer. Figure 14 shows the absorbance of each subject's plasma as a function of time. As seen in the figure, the absorbance values of the plasma of all three subject are not consistent as time goes on, indicating there are some possible interferents in the plasma samples that might react with picric acid. With the absorbance obtained, creatinine concentrations were found using the equation in the calibration curve from figure 5. According to the theoretical clinical value of creatinine level of subject 2, which is 85 μM, this correct creatinine level was only obtained at 52 minutes mark (Figure 15).

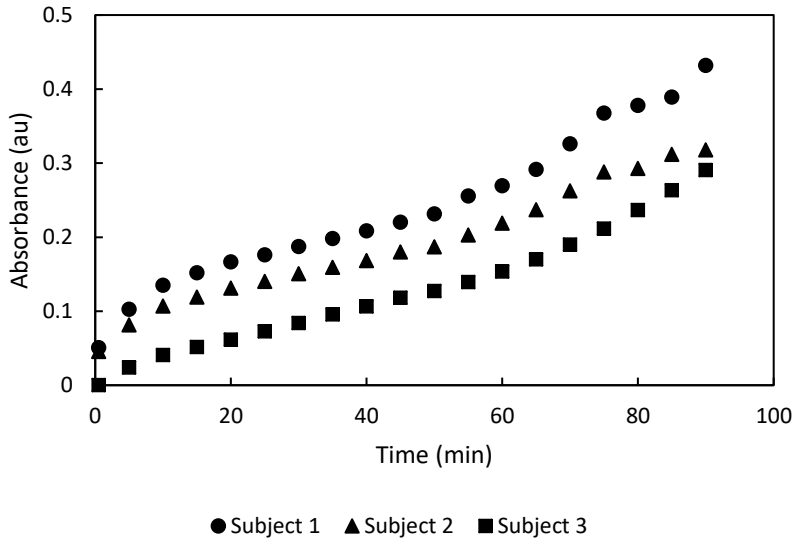

Figure 14: *Spectrophotometric Absorbance of Plasma in Alkaline Picrate Solution of Three Subjects*

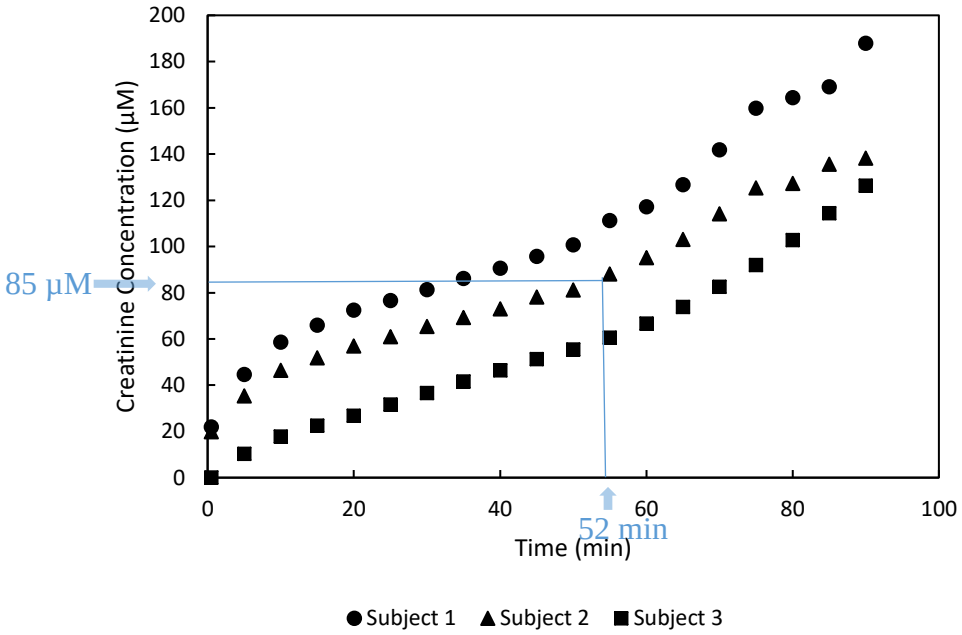

Figure 15: *Creatinine Concentrations Corresponding to the Spectrophotometric Absorbance of Plasma from Three Subjects*

CHAPTER 5

INNOVATIVE PORTABLE RGB READER BOX

5.1 RGB Reader Box

With the developed chemical reaction based for creatinine detection, it was observed in the Results section that the major shortcoming for the sensor to become a PoC device goes to the color differentiation for creatinine quantitation. With the aim of making the sensor more assessable for being a PoC device, there is a need for high sensitive RGB color reader. This chapter introduces the sensitive color reader system which was developed to scan the RGB of the colorimetric sensor.

The reader system uses the RGB sensor (TCS34725) from Adafruit, with an integrated IR blocking filter localized to the color sensing photodiodes[19]. Next to the RGB sensor is the white LED light which turns on right before the system operates to capture the color of the object. The system operated with a micro controller which was fed with an Arduino code to command the system to capture the RGB of the object and displace these values on an LCD. Figure 16a is the image of the RGB reader box with the push button to command the sensor to operate, a port for micro USB power input (figure 16c) and an RGB sensor at the bottom of the sensor box (figure 16d). The sensor strip was modified into a 3D printed PLA filament case with a wick pad embedded in the middle as a sensing area (figure 16e), this wick pad is exposed to the RGB sensor which will sense and displace the RGB output values on the LCD. Figure 16b shows the side of the sensor box with a port for inserting the sensor strips.

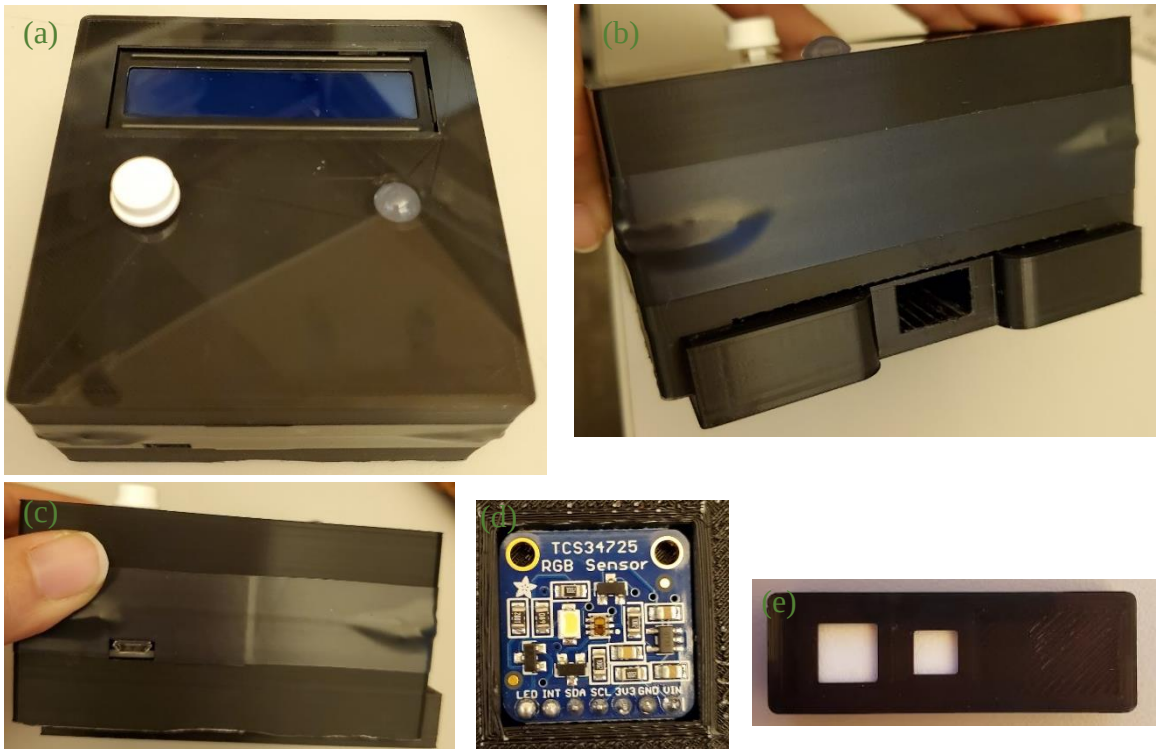

Figure 16: *Picture of (a) RGB Reader Box (b) the Side of the Box with a Port for Sensor Strip (c) Micro USB for Power Supply (d) RGB Color Sensor (e) 3D Printed Sensor Strip*

5.2 Tested Sensor Strips with RGB Reader Box

Creatinine standards were used to test with the developed RGB sensor box. Creatinine from 0 to 200 µM were mixed well with alkaline picrate solution, 50 µL of the creatinine/alkaline picrate mixture were added to an 8 by 8mm wick pad which was embedded inside the 3D printed sensor frame. When the sensor is inserted into the reader, the exposed area of the wick pad with the developed color is facing the RGB sensor chip and the LED light. The system operates when the push button is pressed. RGB values are

then displaced on the LCD in less than a second. Figure 17 shows the intensity of each sensor obtained from reader box's RGB outputs corresponding to different creatinine concentrations.

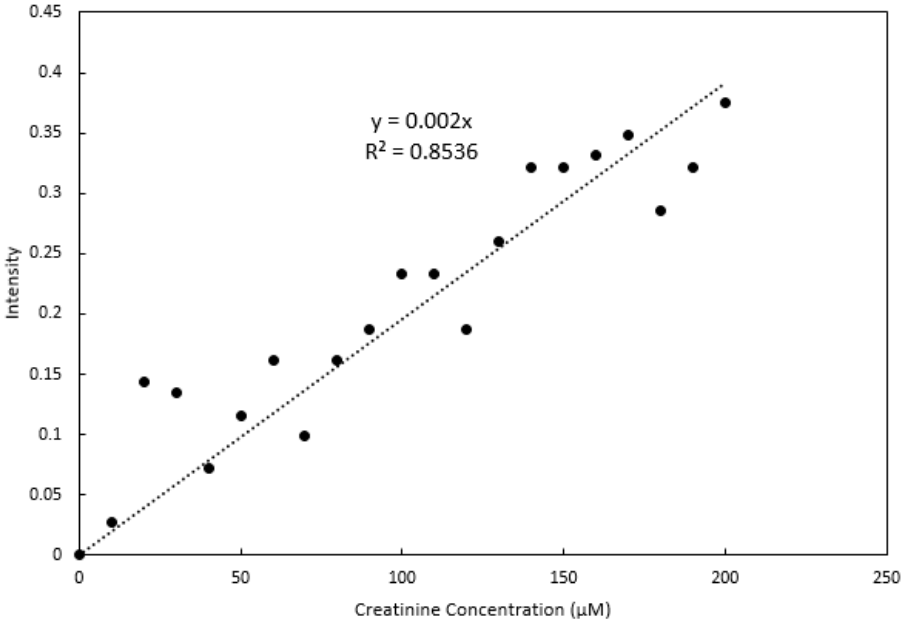

Figure 17: *Intensity Obtained from Sensor Box's RGB Outputs as a Function of Creatinine Concentration*

According to figure 17, intensity obtained from the sensor box has an overall increasing trend correlated to increased creatinine concentration. Even though some data points are quite off from the linear trend, one could tell the difference between creatinine concentrations of 50 µM and 100 µM. The results prove that the system has the potential to become a portable device for sensitive color detection. This system could be further implemented by modifying it to pair with a mobile phone which is designed to systematically calculate the concentration of the analyte with the input RGB values from the RGB reader box. In addition, the design of this reader could be modified to fit any type

of colorimetric sensor to quantify the concentrations of the analytes based on the outputs of the RGB values.

CHAPTER 6

CONCLUSION

With the inspiration from PoC LFA devices for SARS-CoV-2 and pregnancy tests, a new version of LFA was designed and built to detect creatinine based on the analyte-selective chemical reactions method. With the tested creatinine standards, the results obtained from the assay are well correlated to the absorbance from laboratory-based spectrophotometry method with a coefficient of determination of 0.96. However, the main shortcoming of this creatinine sensor is the specificity when it comes to test with plasma sample. The absorbance of the plasma from the spectrum shows an ongoing increasing trend, indicating there could be some possible interferents with creatinine in the plasma samples. The issue with the chemistry is expected to be optimized in the future for more creatinine specificity. Moreover, the proposed RGB reader box is an innovative development to sensitively readout the RGB values of the colorimetric sensors. The reader could be easily modified to pair with any mobile app for easy assessment of any biomarker levels based on colorimetric detection. The idea of using the proposed LFA sensor along with RGB reader box shows a great promise for the development of low-cost PoC biomarker sensor.

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