UNIVERSITY OF CALIFORNIA SAN DIEGO

Utilizing Electrochemical Methods to Determine Reaction Rates on Paper Media

A thesis submitted in partial satisfaction of the requirements for the degree

in

Master of Science in Bioengineering

by

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2022

The thesis of Alex Zhang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego
2022

DEDICATION

To my family giving me the motivation to become one of the first in the family to pursue a higher education.

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ABSTRACT OF THE THESIS

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Biosensors have become a prominent field of research in a world where detection is one of the key methods to prevent spread of disease and obtain medical information in a patient. One of the most cost-effective ways to currently do this involves paper-based analytical devices which provide quick feedback on an analyte of interest. The commonly used method, a lateral flow assay with a colorimetric readout, has currently been one of the key proponents of the field with constant research being done on this subject. However, significant limitations to this method are the sensitivity of the test as well as an immobilization requirement for the reactant, requiring extra steps.

We suggest combining paper-based devices with a previously established readout method, transient induced molecular electronic signal (TIMES), which uses electrochemical signals to determine if a reaction has occurred. By reading the induced charge that comes from the chemical reaction or analyte being observed, immobilization of the analyte can be bypassed. Furthermore, a direct signal can be generated and analyzed to confirm results rather than relying on a weak colorimetric readout. This method will not only lower the amount, and therefore, cost of the material being used, but also simplify the overall process by skipping the immobilization step. In this paper, we use this method to study protein and antibody binding interactions to confirm the validity of this technique. By showing how the results fit to kinetic standards, we can open up the opportunity for further studies on this method.

CHAPTER 1: INTRODUCTION

Molecular-based detection has introduced many platforms over the past few decades from real-time polymerase chain reaction (RT-PCR) to mass spectrometry (MS).[1–3] Most recently, biomolecular detection is of particular interest given the enzymatic reactions that can contribute to a multitude of information such as how fast a reaction takes place or even if a reaction has occurred.[4] Analytical devices have become a fast-developing technology in recent decades to accommodate the advancements in the field.[5]

Paper-based analytical devices (PADs) have been an especially attractive platform to develop due to their cheap cost of manufacturing as well as their ease of operation.[6,7] The most commonly used form of PADs are lateral flow devices given their ease of use and low cost.[8] These devices use the driving force of diffusion to carry the molecules to a capture point to determine the detection of any particles of interest. Generally, this will result in a colorimetric readout indicating that there is a positive result in the test. However, colorimetric tests can be lacking in terms of sensitivity and susceptibility to outside interferences.[9] This can be attributed to light pollution or turbidity of the driving buffer which can cause the end result to be skewed.

An alternative readout method is an electrochemical method, converting the interactions that occur on the paper into data signals that can be more readily

processed. Although still in its nascent phase, electrochemical devices offer more affordable and cost-effective techniques over the more conventional ones.[10] Redox reactive compounds, which are present in metals and small molecules, can be processed and analyzed using different types of voltammetry equipment.[11,12] Cyclic voltammetry is one of the most popular methods to measure current response in redox active reactions by cycling the potential of electrodes between two or more set values.[13]

Current methods of electrochemical detection processes that utilize the PAD system generally involve manipulation of the paper platform.[14] Electrodes that are specifically designed to detect analytes are embedded into the platform. A variety of methods such as inkjet printing and screen printing are used to deliver the electrodes onto the paper.[15,16] However, a major limitation that comes from this method is the fact that the analyte of interest has to be immobilized on the electrode surface using specific methods such as cross-linking.[17,18] This can reduce the effectiveness of the reaction and become time-consuming and costly.

In this thesis, I will present an electrochemical method to utilize with paper surfaces that will bypass the complicated processes of electrode modification or substrate manipulation. This method builds upon previous experiments by utilizing electrodes built into a glass surface which will read the chemical reaction that occurs on a cellulose paper where the chemicals will be added. The paper is pre-wetted beforehand, forming a thin liquid layer, so that the analyte molecules can be gradually diffused together and to increase the solubility of the paper such that the electrode

surface can more easily read the signal. The cellulose fibers in the paper provide mechanical stability with the interconnected structure while still being porous enough to allow the analyte molecules to move through smoothly.[19] After adding the analyte molecules onto the paper, the overall chemical reaction can be detected by its current response and amplified to produce a comprehensible signal.

With the initial stages of this work, we were able to study the binding properties of proteins and antibodies. These two binding reactions were chosen as they are widely used in many fields, but generally not in electrochemical readings. By successfully showing how the reactions will produce signals using this paper-based electrochemical reading, we can open up the possibility of introducing the method to other applications in the future.

1.1 Protein binding

The main interest in the development of pharmaceuticals is how to increase the efficiency of a drug's performance. One of the main ways to control this is to utilize proteins that bind to the medication, effectively enhancing or detracting the drug in question.[20] By investigating the binding properties of proteins, one can determine the best method to increase the effectiveness of the molecules that are meant to be bound.

In this study, we opted to use ninhydrin as our binding agent against amino acids. This commonplace detection method creates a compound known as Ruhemann's purple that would additionally assist us visually on the accuracy of our test. By

measuring the electronic response that occurred while the ninhydrin mixed with varying amino acid concentrations, we were able to determine a consistent trend in the binding mechanism.

1.2 Antibody binding

Another popular binding mechanism utilizes antibodies especially in respect to current events in the world. Generally, a lateral flow test will utilize a sample and capture antigens to determine if the analyte has antibodies of interest. The commonly used antibody, IgG, has a Y-shape that is made of the longer Fc region and the shorter Fab region. There are two types of antibodies: primary and secondary, which are both created in generally the same way - by immunizing a host animal. A primary antibody will specifically bind to a target antigen utilizing an initial host species. Secondary antibodies are created by immunizing a host from a different species with the antibodies from the initial host. This allows for limited cross-reactivity as it will recognize the primary antibody and increase the specificity of binding. The secondary antibody binds to the Fc fragment of the primary antibody, allowing for direct application between the two molecules. IgG specificity lets us characterize the binding kinetics in this specific interaction while allowing us to remain label-free.[21] An image of the binding mechanism is shown in Figure [1].

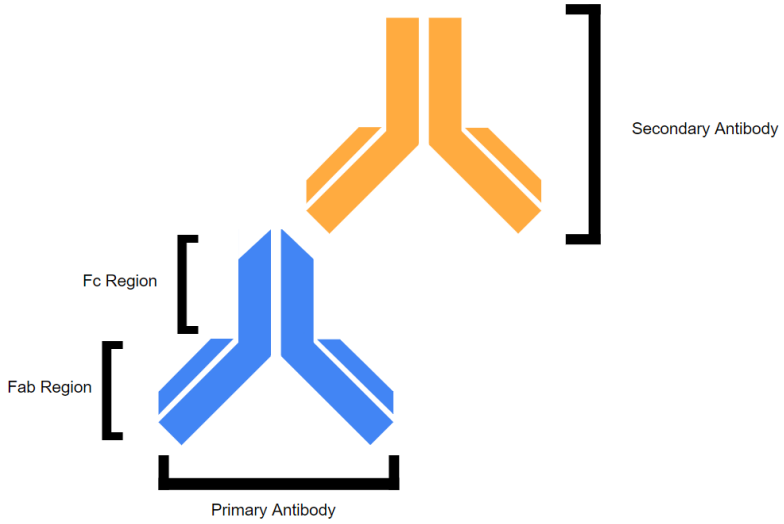


Figure 1: Primary and secondary antibody binding and structure

We opted to use primary and secondary antibody binding in our research as the specific binding allowed for a response without much interference. Without utilizing any blocking agent that would generally need to be used in other tests, we directly measured the binding mechanism between the first and secondary antibodies. Additionally, we were able to measure the signal without having to immobilize the antibodies onto the disposable paper, which cuts the complexity and waste that would be performed in a normal lateral flow assay.

CHAPTER 2: METHODOLOGY

By utilizing the already established transient induced molecular electronic spectroscopy (TIMES) method, we were able to create our own setup using a paper basis as shown in Fig [2]. A 5x20mm^2 filter paper is placed on top of a glass substrate which has two electrodes embedded into the framework. One of the electrodes serves as a sensing mechanism and the other one works as a reference. Depending on the analyte, different types of filter paper may have to be used in order to maximize the efficiency of the measurement since the thickness of the paper affects the fluid absorbance.[22] In our case, we used 750μm thick blotting paper (Whatman^R gel blotting paper) and 180μm 3MM-chromatography filter paper (Whatman^R cellulose chromatography paper) for the ninhydrin and antibody measurements, respectively. The paper was cut to a specific size to match the electrode spacing, but any reasonable size would work so long as the paper was soaked accordingly.

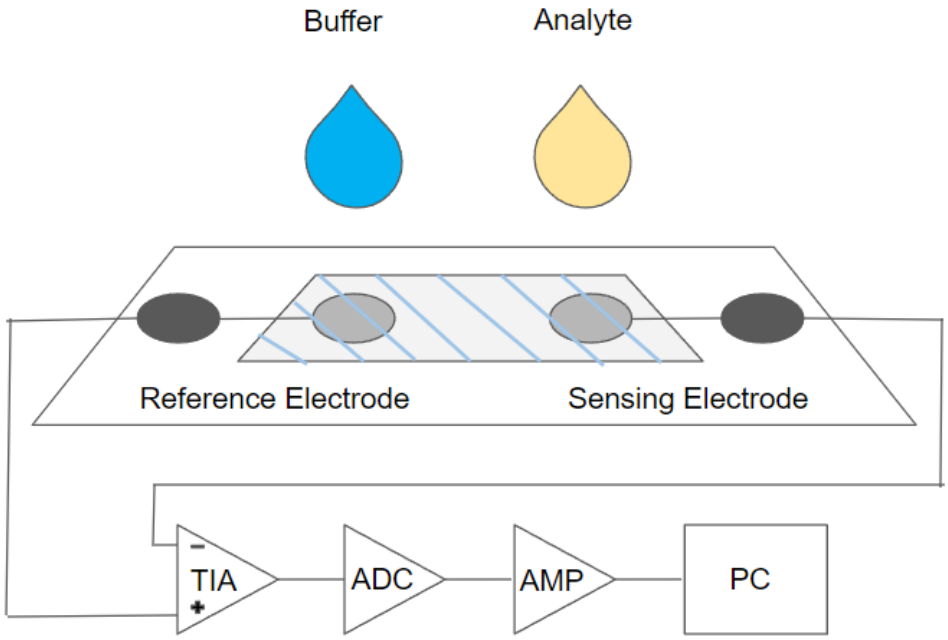


Figure 2: Schematic representation of paper-based TIMES set-up

The filter paper is saturated with a buffer solution in order to ensure that the diffusivity of the chemical analytes is consistent.[23] A fixed volume of the solution was applied to have proper control over the wettability of the paper. This amount of liquid was just enough to form a thin liquid layer on the medium, creating a bridge to the interface of the electrode surface. After a fixed amount of time, the analyte of interest is dropped above the paper where the sensing electrode is located. The initial buffer solution is added onto the area where the reference electrode is located in order to prevent any dilution effect from appearing within the data. The reaction is then placed in a small chamber and set to run for 15min, so no outside interference occurs.

As the reaction occurs, it forms a signal that is captured by the electrodes which were sputtered on with a platinum base. This signal is connected to a transimpedance amplifier (TIA, SR-570, Stanford Research system, Inc., USA), which allows for the input current signal to be transformed into a voltage output where the signal can be intensified using an amplifier. The signal can then be digitized by a data acquisition board (DAQ, USB-6251, National Instrument, USA) and then recorded using a data software (LabView Signal Express). A typical waveform is shown in Fig [3].

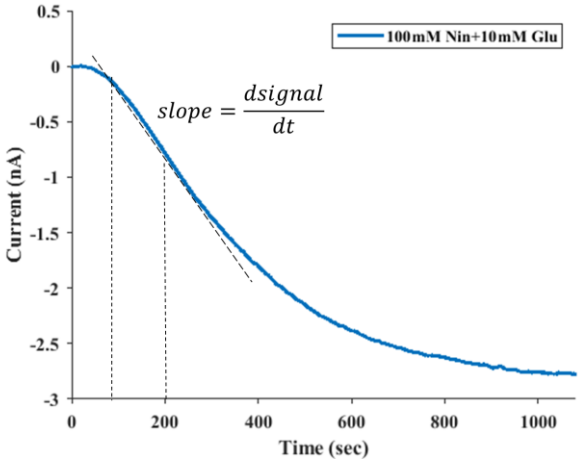


Figure 3: Typical waveform using TIMES method

The signal produced by the chemical reaction follows the format of the previously established TIMES measurements.[24–27] The overall current response is generated from the shift in the surface charge density on the electrode surface due to a change in the charge density of the molecules as more complex molecules are created. Previously this was done in the format of microfluidic devices, but we have now modified the format such that the same circumstances should appear within this paper-based experiment.

By saturating with a buffer solution, we can create the electric double layer that appears when a surface is exposed to liquid. This will create two layers: a bulk or surface layer where ions can flow through freely, and a diffusion layer where ions will pass through as it heads to the positively affected surface charge. The TIMES method will detect the charge difference that occurs as it passes through this diffusion layer.

In this experiment, the charge density changes as the analytes bind together while the reaction occurs on the liquid-solid interface provided by the paper surface. We were able to establish this effect occurring around the 100sec mark where the curve begins to decrease, showing that a reaction is beginning to occur. By changing the initial analyte concentration, it is possible to create different slopes as a result of the of the changing binding molecules, allowing for us to find the kinetics of the complex.

In creating a linear fit model, we designed the experiment such that we can create a reaction kinetic formula. In equation 1, we use two reactants, designated as A and B, with the reaction orders of x and y, respectively. We also establish the reaction constant, k, in the equation. By setting the initial rate as dSignal/dt, we can get the constants necessary to confirm our results by using the slope of the curve as the rate.

$$Initial\ rate\ (slope)\ =\ \frac{d\ Signal}{dt} \propto\ k[A]^x[B]^y \qquad [1]$$

By using a concentration of [A] that's significantly greater than [B], we can manipulate the kinetics such that it becomes a pseudo first-order reaction.[28,29] In doing

so, reactant [B] can be disregarded from the kinetic formula previously stated. Analyzing the overall signal with the new equation 2 allows us to determine the initial reaction rate using the TIMES principle.

$$Initial\ rate\ (slope)\ = \frac{d\ Signal}{dt} \propto\ k[A]^x \qquad [2]$$

2.1 Ninhydrin Experiment

We prepared each solution of either ninhydrin or glutamine, our chosen amino acid for the reaction, with 1X phosphate buffered saline (PBS) solution. Concentrations of 50mM to 200mM were created for ninhydrin whereas concentrations of 0.5mM to 2mM were created for glutamine. We used a fixed amount of 80μL of ninhydrin to soak the paper each time. After waiting a period of time for the paper to become saturated, 2μL of glutamine was added where the sensing electrode was located and 2μL of 1X PBS buffer was added where the reference electrode was located.

2.2 Antibody Experiment

We prepared our primary and secondary antibodies by first choosing the polyclonal antibodies to use. It should be noted that most studies would most likely just use antibody-antigen binding kinetics given their close specificity, but the use of primary and secondary antibody binding was decided to determine their usage in lateral flow tests. Additionally, while most studies utilize monoclonal antibodies in their experiments,

polyclonal antibodies were chosen as they allow for multiple binding sites which helps to increase the sensitivity and amplify the overall signal.[30] The primary antibody, goat anti-rabbit IgG, is applied onto the paper initially while the secondary antibody, donkey anti-goat IgG will be pipetted onto the paper after saturation is complete.

The setup for this experiment is slightly different to ensure even immobilization of the antibodies. Although our method does bypass the necessity of any special materials or methods to immobilize the antibodies, there is still an initial requirement for the antibodies to be physically absorbed into the cellulose paper. The secondary antibodies can then flow through in the second step and bind accordingly which is not dependent on the orientation of the primary antibody. On dry filter paper, 2μL of the primary antibody is spotted on and left to fully dry. Afterwards, it is moved to the electrode plate such that the immobilized spot rests where the sensing electrode is located. 40μL of 1X PBS is used to saturate the paper and then 2μL of the secondary antibody is pipetted onto both the sensing and reference sites.

2.3 Surface Plasmon Resonance (SPR)

In order to determine the affinity kinetics of the antibodies, we sent our materials to the Scripps Research facility where they operated the Biacore 2000 to conduct a SPR measurement. They gave us results that covered binding kinetic constants as well as the raw data so we could compare it with our own research. The raw data is shown in the supplementary section. Surface plasmon resonance is one of the current best

methods to determine the kinetics of monoclonal and polyclonal antibodies given the efficiency of the machine as well as the depth of information that it can give.[31,32] In our specific case, we mostly accounted for the Kd of the reaction which we could calculate by doing a linear fit model with the data that was given to us. Data analysis was performed by measuring the tangent of the slope where the signal first shows a reaction, implying that the binding has started to occur.

2.3 Acknowledgement

Chapter 2, in part, is a reprint of the material as it is to be published later: Chi-Yang Tseng, Alex Zhang, Yu-Hwa Lo. Paper-Based Transient Induced Molecular Electronic Signal (TIMES) on Reaction Kinetic Study. The dissertation author was the first author of this paper.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Ninhydrin Experiment

Starting with ninhydrin, a number of concentrations were formulated of every mixture for two separate experiment sets. Firstly, it was important to set up the control which we established using both PBS and glucose. They were both used as a way to ensure that ninhydrin was reacting to the amino acid accordingly instead of reacting to any analyte added onto the sensing electrode. As shown, the signal shows little to no reaction where any change in signal is most likely due to some residual dilution effects that could not be avoided. Overall, the response is within expectations and can be ignored.

In determining the kinetics for this experiment, it is important to note the actual chemical reaction that takes place when ninhydrin interacts with the α-amino group of primary amino acids.[33] A simplified version of the reaction is shown in Figure [4].[34] The overall reaction has a rate-determining step in which a nonprotonated amino group must cause a nucleophilic-type displacement of a hydroxyl group in the ninhydrin hydrate. By completing this step, ninhydrin and the amino acid will undergo a reaction that creates the following products: an aldehyde, ammonia, hydrindantin, CO_2, and Ruhamann's purple. Essentially, two ninhydrin molecules are required per one one molecule of amino acid in order to complete the reaction. For this reason, pseudo first-order rate constants are divided in half to create second order constants. [35,36]

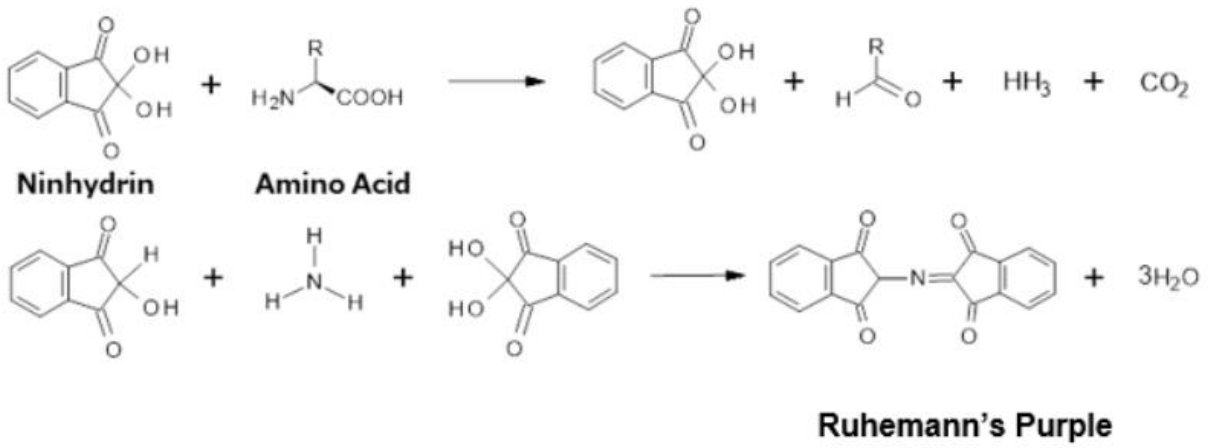


Figure 4: Simplified mechanism of ninhydrin reacting with amino acid

The first experiment set involved changing the concentration of the amino acid, glutamine, while holding the concentration of ninhydrin at a fixed value. By running through the different glutamine concentrations: 0.5mM, 0.7mM, 1mM, 1.4mM, and 2mM, we were able to overlay the response signals as shown in Figure [5]. After about 100sec, the reaction starts to take place causing a dip in the signal as the electrodes sense the charge density changing. Eventually, the current response should go back to the baseline of 0 mA as the reaction reaches equilibrium and no other reaction takes place. However, given that this would most likely require a significant amount of time as well as the possibility that outside interference could occur such as the paper drying up, we decided to set the recording duration to be 15min. The slopes within the first 100-200sec were used to calculate the initial reaction rate via linear fitting. The concentrations used for each analyte can be found in Table [1] along with the initial rate calculated. The ratio between each concentration is approximately 1.4 which is in line with the ratio between the initial rates as well. This shows that the reaction rate is

linearly proportional to the concentration of the amino acid, showing how it is first order in respect to glutamine.

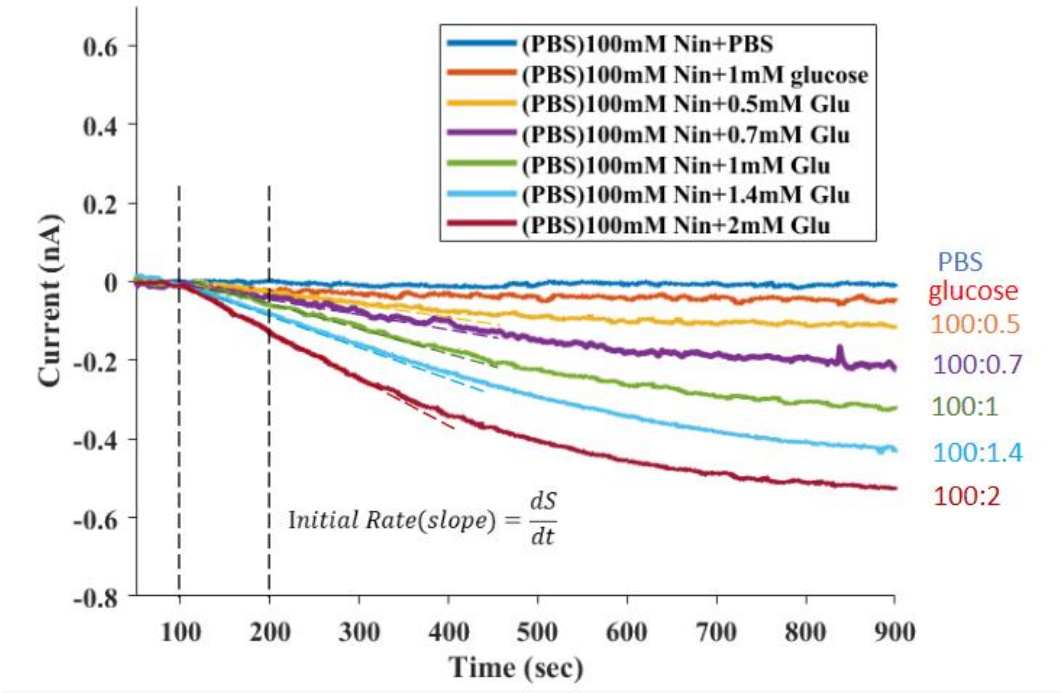


Figure 5: Overlapped signals of ninhydrin reacting with increasing concentrations of glutamine over time

Table 1: Ninhydrin reacting with increasing concentrations of glutamine with calculated initial rate of reaction

Ninhydrin	Glutamine	Initial Rate (dS/dt) (slope 100-200s)	Slope ratio
100mM	0.5mM	-2.673 x10^{-4}	1.43
100mM	0.7mM	-3.832 x10^{-4}	1.58
100mM	1mM	-6.087 x10^{-4}	1.31
100mM	1.4mM	-8.015 x10^{-4}	1.52
100mM	2mM	-12.239 x10^{-4}	
	(~1.4x increasing)		**Avg: 1.46**

The second experiment set involved the opposite condition in which the concentration of glutamine was held constant while the concentration of ninhydrin was changed. The overlapped signals are shown in Figure [6] where the varied ninhydrin concentrations for this set: 50mM, 70mM, 100mM, 140mM, and 200mM, can be found in Table [2] along with their calculated initial rate values. As shown, similar ratios are found when compared to the first experiment set. This also serves to validate the fact that the reaction rate is proportional to the ninhydrin concentration given that we manipulated the reaction to become pseudo first-order.

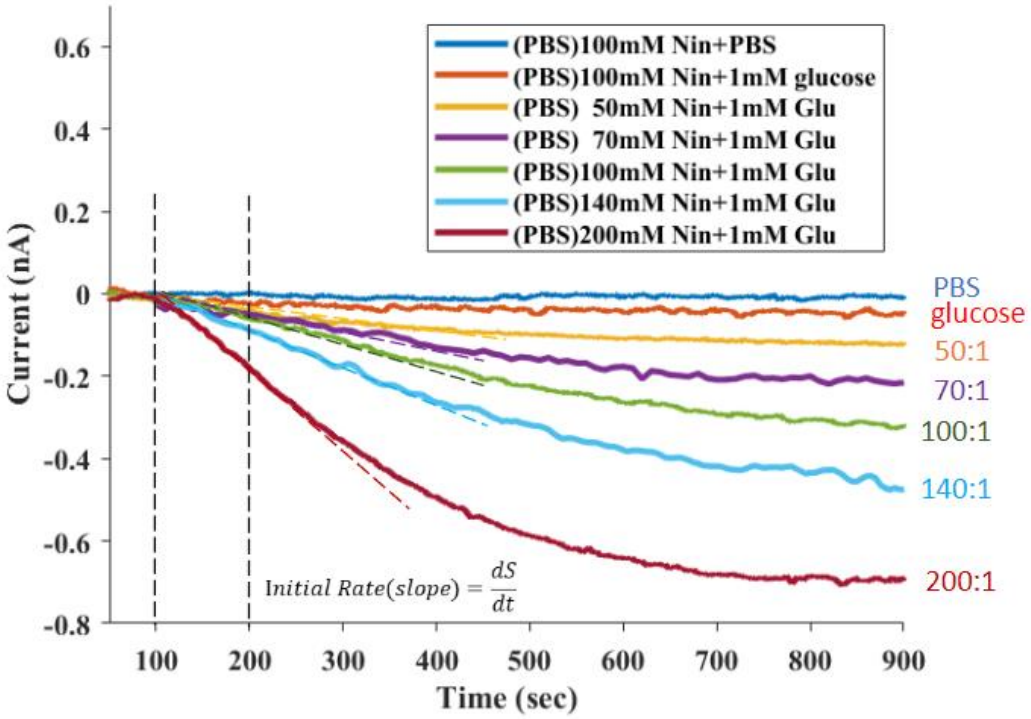


Figure 6: Overlapped signals of increasing concentrations of ninhydrin reacting with glutamine over time

Table 2: Increasing concentrations of ninhydrin reacting with glutamine with calculated initial rate of reaction

Ninhydrin	Glutamine	Initial Rate (dS/dt) (slope 100-200s)	Slope ratio
50mM	1mM	-2.778×10^{-4}	
70mM	1mM	-3.736×10^{-4}	1.34
100mM	1mM	-6.087×10^{-4}	1.63
140mM	1mM	-9.312×10^{-4}	1.53
200mM	1mM	-16.513×10^{-4}	1.77
(~1.4x increasing)			**Avg: 1.56**

By using our established rate equations, we can more precisely confirm the kinetics, $initial\ rate\ (slope) = \frac{d\ Signal}{dt} \propto k[A]^x$ where in experiment set 1, we use the modified pseudo-first order equation such that [A] = [Glu]. By keeping the ninhydrin value constant, we can ascertain that any changes come from the changes in the amino acid concentration. As such, rate = k'$[Glu]^x$ where k' = k_{on}[Nin] and x = 1, given the consistency in the initial rate. In experiment 2, we use ninhydrin as the variable such that rate = $k_{on}[Nin]^y[Glu]$ where [Glu] = 1, given the values used, and y = 1, as the rates are also proportionally consistent. Essentially with these two experiments, we show that the rate = k_{on}[Nin][Glu]. Overall, a second order reaction can be determined which is consistent with previous studies.[35]

3.2 Antibody Experiment

In the antibody experiment, a slightly different process was used to create our results given the structure of secondary and primary antibodies. With the binding mechanisms of antibodies, a basic immobilization step had to occur before proceeding forward with the experiment. Figure [7] shows the overlaid response signals for this experiment. The control for this experiment was done by simply adding the secondary antibody onto the saturated paper, excluding the initial primary antibody immobilization. This allows us to test whether the secondary antibody produces any reaction with the paper or the buffer material even with a lack of anything to bind to. As shown in the figure labeled as the dark blue line, there was little reaction which is negligible supposing that its attributed to evaporation of the paper or unwanted air particle effects. This allows us to move onto the main experiment, choosing different concentrations to use in our setup. The list of varied concentrations in the secondary antibody: 0.25mg/ml, 0.35mg/ml, 0.5mg/ml, 0.7mg/ml, and 1mg/ml, are shown in Table [3] along with the determined initial rates.

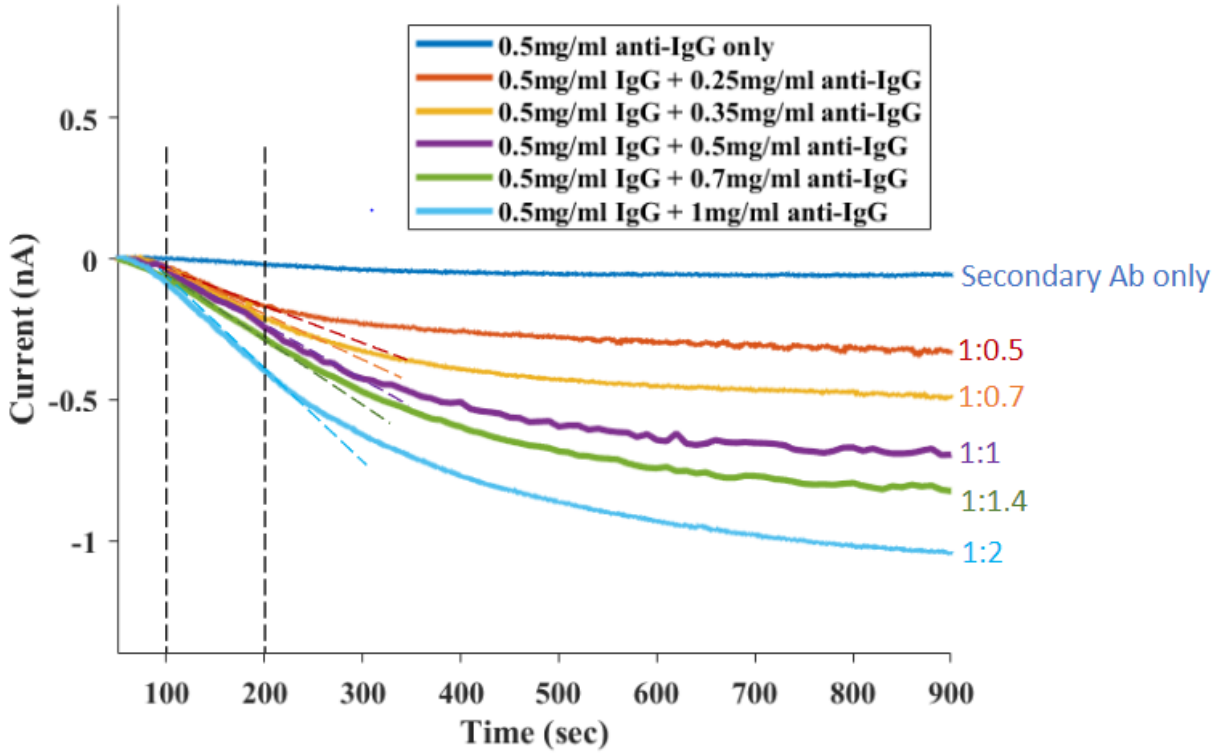


Figure 7: Overlapped signals of primary Ab reacting with increasing concentrations of secondary Ab

Table 3: Primary Ab reacting with increasing concentrations of secondary Ab with calculated initial rate of reaction

1st IgG Ab	2nd IgG Ab	Initial Rate (dS/dt) (slope 100-200s)	Slope ratio
0.5 mg/ml	0.25 mg/ml	-14.39×10^{-4}	1.26
0.5 mg/ml	0.35 mg/ml	-18.13×10^{-4}	1.06
0.5 mg/ml	0.5 mg/ml	-19.13×10^{-4}	1.14
0.5 mg/ml	0.7 mg/ml	-21.75×10^{-4}	1.43
0.5 mg/ml	1 mg/ml	-31.19×10^{-4}	
	(~1.4x increasing)	$* \sqrt{1.4} = 1.18$	**Avg: ~1.22**

Similar to the ninhydrin experiment, initial rates were obtained via linear fitting of the slope for the first 100-200sec. The concentration of the secondary antibody goes up by a ratio of 1.4 times while the ratio between each concentration increases by a factor of 1.2 times, a close approximation to the square root of 1.4. With this data, we can surmise that the binding kinetics is first order in the primary antibody and a half order in the secondary antibody. This half-reaction order is most likely explained by the multivalency of polyclonal antibodies.[37] Using SPR analysis, we confirmed the binding kinetics of the antibodies, where the measured overlapped signals are shown in Figure [8].

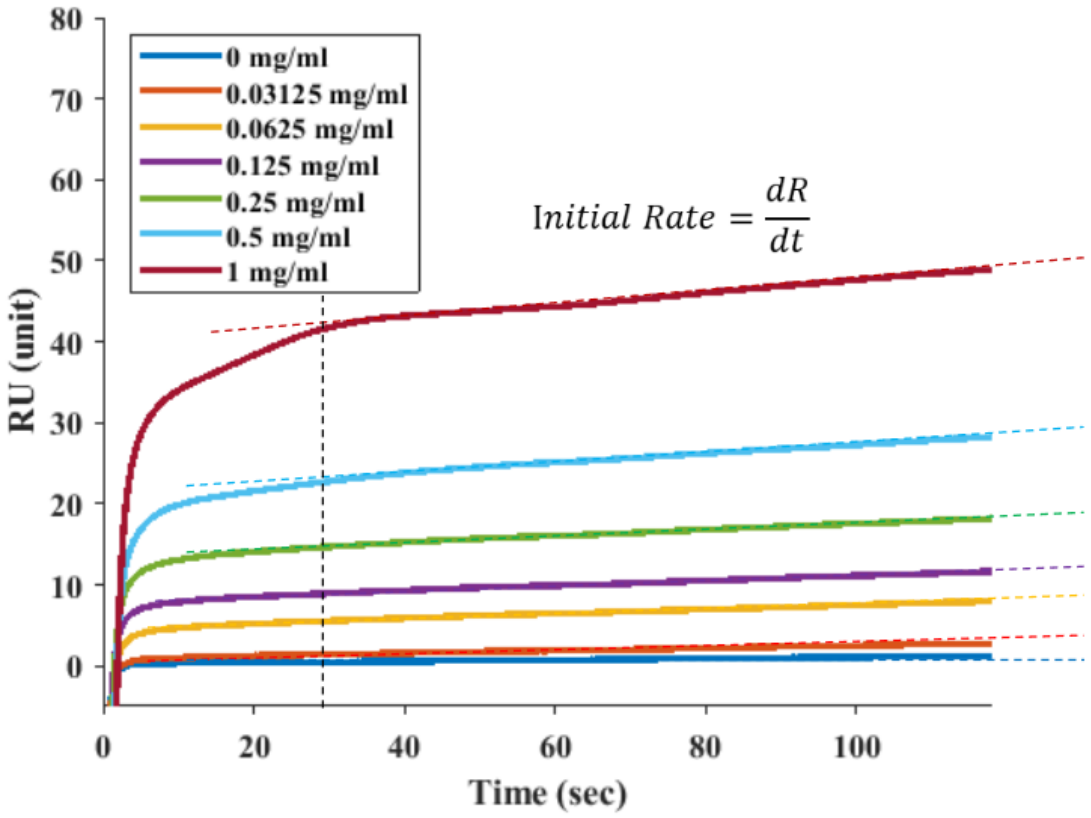


Figure 8: Overlapped signals of SPR measurement

The calculated initial rate values from the SPR are shown in Table [4]. The initial binding rate was measured by using the tangent of the slope where the strongest association occurs.[38] Considering our initial equation [2], $initial\ rate\ (slope) = \frac{d\ Signal}{dt} \propto k[A]^x$, we can show that from both the TIMES experiment and the SPR results, the average initial rate ratio is a close approximation to the square root of the concentration of the analyte:

$$Initial\ rate = \frac{d\ Signal}{dt} \propto k[A]^{1/2}$$ where [A] = secondary antibody

Mirroring the square root ratios, we show that the overall reaction is a one and a half reaction order and can confirm our antibody results.

Table 4: Calculated SPR ratios with calculated initial rate of reaction

1st IgG Ab	2nd IgG Ab	Initial rate (dR/dt) (slope ~80s	Slope ratio
0.5 mg/ml	1 mg/ml	0.07654	1.35
0.5 mg/ml	0.5 mg/ml	0.0568	1.44
0.5 mg/ml	0.25 mg/ml	0.03939	1.27
0.5 mg/ml	0.125 mg/ml	0.03111	1.12
0.5 mg/ml	0.0625mg/ml	0.02776	1.79
0.5 mg/ml	0.03125 mg/ml	0.01553	1.86
0.5 mg/ml	0 mg/ml	0.008332	
	(2x increasing)	$* \sqrt{2} = 1.41$	**Avg: ~1.47**

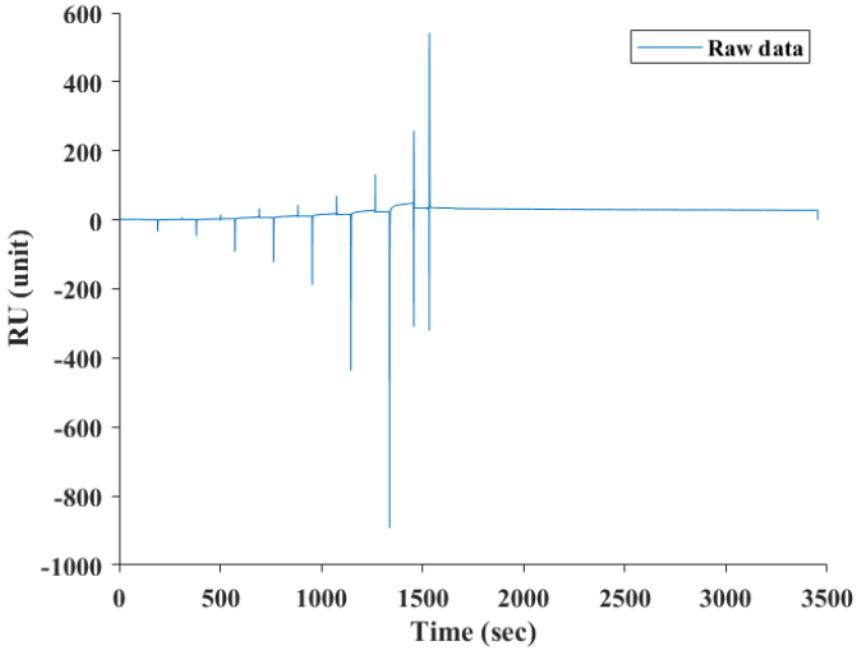
3.3 Acknowledgement

Chapter 3, in part, is a reprint of the material as it is to be published later: Chi-Yang Tseng, Alex Zhang, Yu-Hwa Lo. Paper-Based Transient Induced Molecular Electronic Signal (TIMES) on Reaction Kinetic Study. The dissertation author was the first author of this paper.

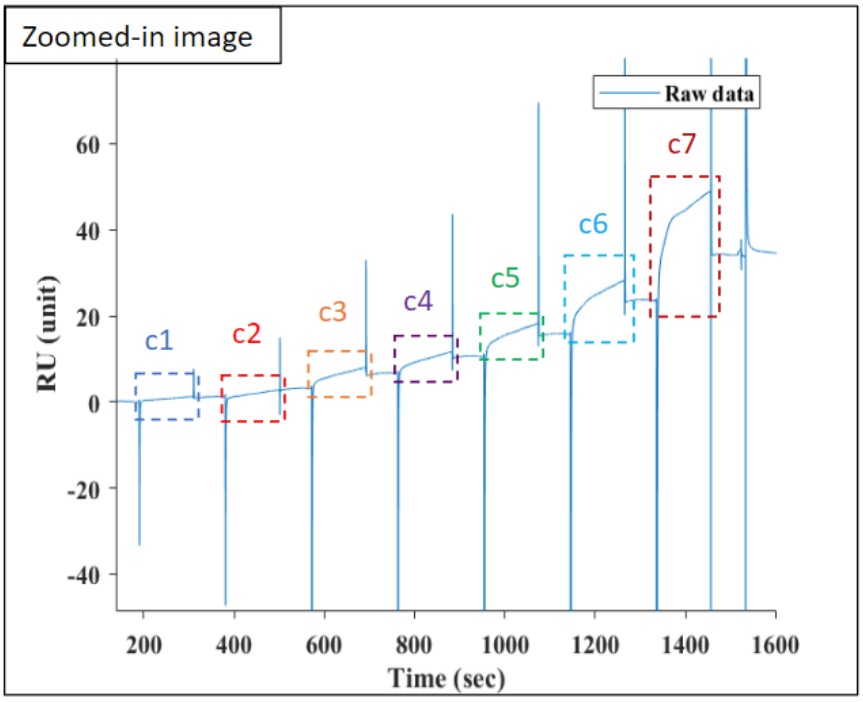
CHAPTER 4: CONCLUSION

Through these experiments, we have learned that the TIMES method can be applied to a paper-based method. The molecular interaction can be transposed into a digital signal that can then be further analyzed to understand the kinetics of the analytes. There can still be changes to be made in order to fully develop this system such that a consistent and clear signal can be shown. Further research can be applied on the signal itself to calculate other kinetic constants or perhaps other information that chemical reactions can give. Nevertheless, the current system shows promising results and opens up the potential for application to other detection systems such as lateral flow assays. This paper-based method can provide a quick and efficient way to cut manufacturing and labor costs given further development.

SUPPLEMENT



Sup. Figure 1: Raw data of SPR measurement



Sup. Figure 2: Zoomed in close-up of the raw data showing the signals that were overlapped

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