POINT-OF-CARE BODY FLUID DIAGNOSTICS

IN MICROLITER SAMPLES

by

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Dedication

To my parents and my wife, Yu-Ting Lin, for their love and support

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Point-of-Care Body Fluid Diagnostics

in Microliter Samples

Abstract

by

LINUS TZU-HSIANG KAO

Abstract

Point-of-care testing (POCT) generally refers to the implementation of laboratory tests near the patient with the goal to minimize turnaround time, reduce medical cost, and improve medical outcome. The aim of this research is to develop, validate, and optimize a preliminary POCT system for body fluid diagnostics using our existing analytical platform, Rotating Sample System (RSS). The RSS is a convective platform for different optical and electrochemical analyses in microliter-sized samples.

In this work we have developed a reagent-free electrochemical micro pH-stat for *in vitro* enzyme assays where a titrant of acid or base is produced by water electrolysis on the RSS platform. As water electrolysis induces no volume change and the current that generates the reagent can be precisely measured even at low levels, very small samples in

the 1-20 μL volume range become accessible for pH-stating: a reduction of more than an order of magnitude in specimen size relative to the most conventional methods. More importantly, analysis using untreated biological sample is feasible as turbidity would not influence pH-stat measurement. The RSS micro pH-stat effectively operates as a galvanostat with an output range of ±0.1-100 μA and can withstand sample impedance up to 100 kΩ. The cathodic current efficiency is virtually 100% in both buffer and serum samples. Results of cholinesterase activities in both buffer and serum using the proposed reagent-free pH-stat have been validated with standard optical techniques ($r^2 \geq 0.97$). This novel technique hence has great potential to become a miniaturized analyzer for point-of-care diagnostics.

Chapter 1

Introduction of Point-of-Care Testing, Clinical Enzymology, and Electrochemical Enzyme Assays

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1.1. Introduction

Rudimentary point-of-care testing (POCT) was described as early as 1883 in London (Kiechle 2002). POCT generally refers to the implementation of laboratory tests near the patient's bedside or at the "point of care" with the goal to minimize turnaround time, reduce medical cost, and improve patient management. Due to its potential to reshape the delivery of medical service, POCT is an emerging modality growing rapidly in the healthcare field. Our long term goal is to develop a portable point-of-care device for an array of body fluid analyses to aid biomedical research and expedite clinical diagnostics. The aim of this thesis is to develop, validate, and optimize a preliminary POCT platform using reagent-free micro pH-stating techniques for universal enzymatic analysis in body fluids.

Body fluids such as blood continuously circulate throughout the body, delivering nutrients to cells and transporting products from and toward tissues. They contain a massive amount of information about the functioning of all tissues and organs in the body. Consequently, accurate, fast, and affordable techniques to extract useful information from cellular and molecular components in body fluids are of prime interest for medicine and research. Achieving this goal requires not only the understanding of biology but also adequate technologies.

The target molecules that are analyzed in this work are enzymes. Enzymes are proteins catalyzing a large variety of biochemical reactions in the body. Changes in enzyme activity in body fluids are often utilized as diagnostic markers for diseases, tissue injuries, and toxic exposure. Therefore enzyme assays are one of the most important

procedures for both biomedical research and clinical testing. Currently most enzyme assays of body fluids are performed using spectrophotometry that requires several pre-treatment steps. Here we introduce an innovative electrochemical pH-stating platform that promises the potential of performing a wide spectrum of enzyme assays in a simpler, quicker, and more accurate fashion.

Electrochemical techniques in general are well-suited for point-of-care diagnostics due to the ability of operation even in untreated biological samples. In addition, incorporation of circuits and electrodes into microfabricated chips is straightforward and thus a fully integrated, portable device can be achieved. The following sections of this chapter will introduce in detail the concept of point-of-care testing, the background of enzymology and enzyme assays, and finally the rationale and the advantages of using electrochemical techniques for developing our point-of-care system for enzymatic analyses.

1.2. Point-of-Care Testing

In the early time, medical diagnosis was made by performing simple tests close to the patients along with the information gained from the doctor's observation of the patient's symptoms and previous medical history. Nowadays it is estimated that 60-70% of medical decisions are depended on the measurement of chemical parameters in biological samples (Forsman 1996). With scientific progress in identification of new disease markers and the development of advanced analytical technologies, the majority of

today's diagnostic tests have moved away from the patient-side and performed in centralized clinical laboratories using sophisticated analytical systems.

Even though the analytical procedures are mostly automated, significant time constraints occur in the sample transportation from the site of patient care to a central laboratory as well as the waiting time in the sample queue. It usually takes hours or even days to issue the test result back to clinician that poses a major delay in making treatment decisions (Figure 1.1). Apart from the time delay, studies showed that over 80 percent of laboratory testing errors do not occur during testing but during pre- and post-analytical procedures such as sample mismatch, misidentification of the requested test, and inappropriate specimen collection and handling procedures (Plebani 2006). Therefore, isolation of these facilities from the site of patient care has hindered the diagnosis as well as demanded relatively high labor need in the hospitals.

Point-of-care testing (POCT) is generally defined as any testing performed near the patient with a goal of reducing overall turnaround time and improving medical outcome (Price, St John et al. 2004). The settings where POCT is performed include, for example, doctors' offices (e.g., primary care facilities and outpatient clinics), emergency departments, intensive care units, operating rooms, patient transport settings, mobile nursing practices, workplaces, homes, and some more extreme conditions such as settings in military operations and even space shuttles (von Lode 2005). As suggested in Figure 1.1, adopting POCT in the hospital setting would not only expedite the process of laboratory testing, but also eliminate many manual operations (such as sample labeling, transporting, result reporting) which potential medical errors could arise from. Hence

POCT has acquired increasing attention and has the potential to reshape the delivery of medical services.

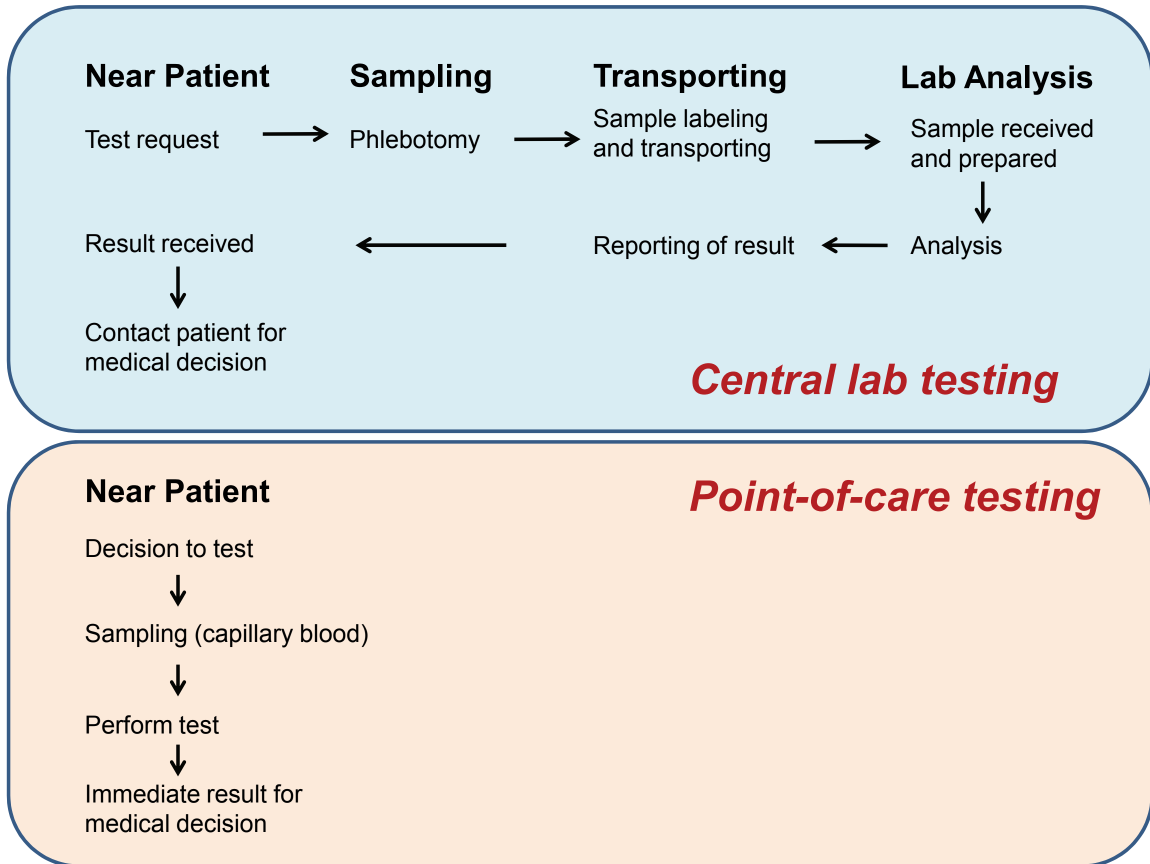


FIGURE 1.1. Comparison of the clinical testing process in an outpatient situation between the central laboratory and POCT methods. The processes are shown in a simplified format and sometimes additional steps may be required (von Lode 2005).

1.2.1. Current state-of-the-art

Here we describe some of the most commonly used POCT instruments and the enzyme assays that they can perform are highlighted:

1. Abaxis Piccolo xpress The Piccolo xpress is a workstation type of clinical chemistry system, roughly the size of a shoebox, designed for on-site patient testing. 0.1 cc of whole blood, serum or plasma can be added directly to the patented 8 cm diameter single use plastic disc containing the liquid diluent and dry reagents for up to 15 simultaneous tests. The disc is placed in the analyzer drawer where centrifugal and capillary forces are used to separate plasma from whole blood, and mix the reagents with sample in the disc. The Piccolo xpress monitors the reagent reactions simultaneously using optical detection and calculates the results in approximately 12 minutes.

The enzyme assays that Piccolo xpress can deliver are: *creatine kinase, alkaline phosphatase, gamma glutamyltransferase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, amylase* (Abaxis).

2. Abbott i-STAT i-STAT is a battery-operated handheld analyzer that uses whole blood for testing. The i-STAT analyzer performs tests via single-use disposable cartridges that are smaller than a business card and packed with biosensor technology and advanced microfluidics. Testing is performed by administering two drops of blood to the cartridge which is then closed and inserted into the analyzer. The solid-state chips contain

micro-fabricated thin film electrodes configured to perform specific tests with chemically sensitive membranes and films containing reagent chemicals.

The only enzyme test that i-STAT can perform is *creatine kinase* for myocardial infarction and the assay time takes about 5-10 minutes (Abbott).

3. Diavant Reflotron Plus The Reflotron Plus is an in vitro diagnostic device designed for the determination of 17 clinical parameters using test reagent strips. This workstation type of instrument operates on the principles of reflectance photometry. The incorporation of a plasma-separating system makes it possible to use capillary or venous whole blood as well as serum and plasma. The required testing time is about 2-3 minutes.

Enzymes can be tested by this device are *alkaline phosphatase, amylase, aspartate aminotransferase, alanine aminotransferase* (Diavant).

Compared to the available technologies, our approach has the potential to analyze a broader range of enzyme markers such as cholinesterase, urease, lipase, glutamate dehydrogenase and all other hydrolases.

1.3. Fundamentals of Enzymology

Enzymes are proteins that are excessively present in living organisms and act as catalysts in many biochemical reactions. They are indispensable for cellular regulation, metabolic activity, and signal transduction. In our body, several different enzymes work together in a specific order to form metabolic pathways. Within the pathway, one enzyme takes up the product of another enzyme as a substrate. After the catalytic reaction, the resulting product is further passed on to another enzyme (Figure 1.2). Consequently, the catalytic activities of individual enzymes are directly linked to the kinetics of whole biochemical chain reactions. For example, the urea cycle shown in Figure 1.2 which removes ammonia from our body is composed of five catalytic enzymes. Deficiency or loss of activity in any of the first four enzymes in the cycle results in the accumulation of ammonia. Since no effective secondary clearance system for ammonia exists, disruption of this pathway results in the rapid development of hyperammonemia symptoms (Batshaw 1984).

Assay of enzyme activity is therefore one of the most frequently performed procedures in biochemical and clinical laboratories. Biochemical studies of enzyme assays involve detection and identification of enzymes, estimation of the amount of enzyme present, defining its functional roles and characteristics, investigation of enzyme inhibition and activation, and studies of related genetic disorders.

Enzymes can also be used as analytical tools for quantification of many substances with high sensitivity and selectivity. The advantage of possessing high specificity for the substance being determined largely eliminates the time-consuming and

laborious procedures of preliminary sample separation and purification. Therefore, an assay of a specific enzyme can be directly carried out on complex biological mixtures such as blood or serum. For instance, glucose oxidase has been widely utilized in both solution and immobilized forms for measuring glucose concentration of diabetic patients (Foulds and Lowe 1986; Zhan, Seong et al. 2002).

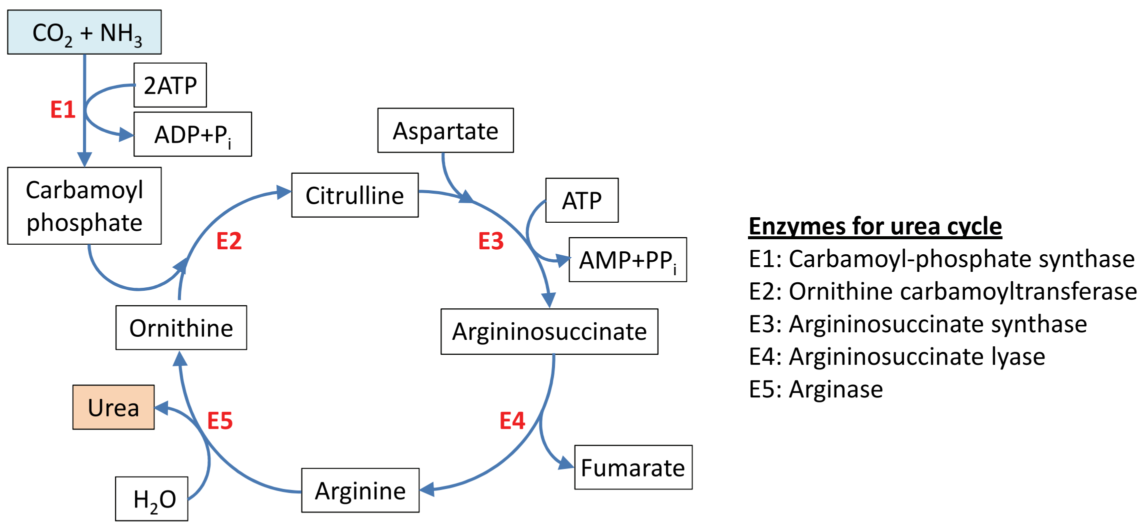


FIGURE 1.2. Urea cycle: the primary mechanism for disposal of waste nitrogen resulted from protein turnover and dietary intake. The first two enzymatic reactions leading to the synthesis of urea occur in the mitochondria, whereas the remaining three enzymes in the cycle are located in cytoplasm.

1.3.1. Significance of clinical enzyme analysis

Enzymes are useful clinical markers for tissue damages, diseases, and toxic exposures. Measurement of enzyme content in blood plasma or serum often provides valuable insights into the underlying metabolic processes and physiological conditions. Injury to tissue releases cellular substances to the circulatory system by capillary or lymphatic transfer. The interest of clinical diagnostics lies in measuring the activity of enzymes which exist predominantly in intracellular matrices and are normally present low activity in serum. Due to the facts that intracellular enzyme concentration is usually three to four orders of magnitude greater than that in the extracellular fluid, and extremely small amount of enzyme can be detected by its catalytic activity, the elevation of enzyme activity in serum becomes an extremely sensitive diagnostic marker of even minor cellular damage (Bais and Panteghini 2006). Therefore by measuring abnormally elevated activities of tissue-specific enzymes, the location and pathological nature of tissue damage can be identified. As an important example, acute myocardial cell response to ischemia releases high concentration of creatine kinase (CK-2) to the plasma. Consequently, increases of CK activity in serum is closely related to acute myocardial infarction (Ishikawa, Saffitz et al. 1997). Lipase (LPS) measurement in serum is used to diagnose acute pancreatitis. After an attack of acute pancreatitis, the activity of serum LPS increases within 4 to 8 hours, peaks at about 24 hours, and decreases within 8 to 14 days (Tietz and Shuey 1993).

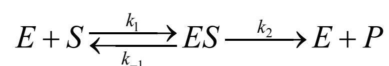
Apart from tissue-specific necrosis, abnormal elevation or reduction of enzyme activity in serum can also contain important pathological information. For example, the activity of serum cholinesterase (ChE) has served as a sensitive indicator of synthetic

capacity of liver. A 30-50% drop in ChE activity is observed in acute and chronic hepatitis. Decreases of 50-70% occur in advanced cirrhosis and carcinoma of the liver. Besides, organic phosphorus compounds presented in many insecticides, such as parathion, sarin, and tetraethyl pyrophosphate, also inhibit activity of ChE. Hence the decay of ChE activity in serum is also an early diagnostic sign of possible insecticide poisoning (Mcqueen 1995).

Because of the significance of enzyme assays in disease diagnosis, a simple and rapid screening tool that can avoid current lengthy procedures in clinical practice would be extremely beneficial. For the purpose of data validation, we chose cholinesterase as a model enzyme for testing our reagent-free micro pH-stat system.

1.3.2. Enzyme Kinetics

The enzyme catalytic reaction is initiated by binding substrate (*S*) to the active site of enzyme (*E*). The free energy released by the formation of an enzyme-substrate complex (*ES*) transforms the substrate to its activated state. The activation takes place with less external energy so that the energy barrier (ΔG) of the reaction is lowered and the breakdown process is accelerated. The ES complex breaks down to the product (*P*) and free enzyme. The general enzymatic reaction can be described as:



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P$$

Theoretically, reactions catalyzed by enzymes are reversible. However, often times the reaction is going more rapid in one direction than the other. The kinetics of the dominated reaction can be so rapid that some of those reactions are considered irreversible. Declines of reaction kinetics due to approaching the equilibrium can be prevented by using consecutive enzymatic reactions for product removal. Enzyme kinetics is commonly expressed by the Michaelis-Menten equation (Eq. 1.1) with the following assumptions: (1) no product is present at time zero so that there is no backward reaction, (2) equilibrium is attained rapidly among E, S, and ES complex, and (3) concentration of the ES complex is negligible compared to the total substrate concentration:

$$v = \frac{V_{\max}[S]}{K_m + [S]} \tag{1.1}$$

where v is the overall rate of reaction, $V_{\max}$ is the maximum reaction velocity, and K_m is the Michaelis-Menten constant defined as:

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{1.2}$$

The Michaelis-Menten equation correlates the kinetics of an enzyme-catalyzed reaction to substrate concentration. The value of K_m is given by the substrate concentration at which one half of the maximum reaction rate is obtained. The maximum velocity of the reaction ($V_{\max}$) is a theoretical value which corresponds to the hypothetical situation when all enzyme molecules are saturated with substrate (Figure 1.3).

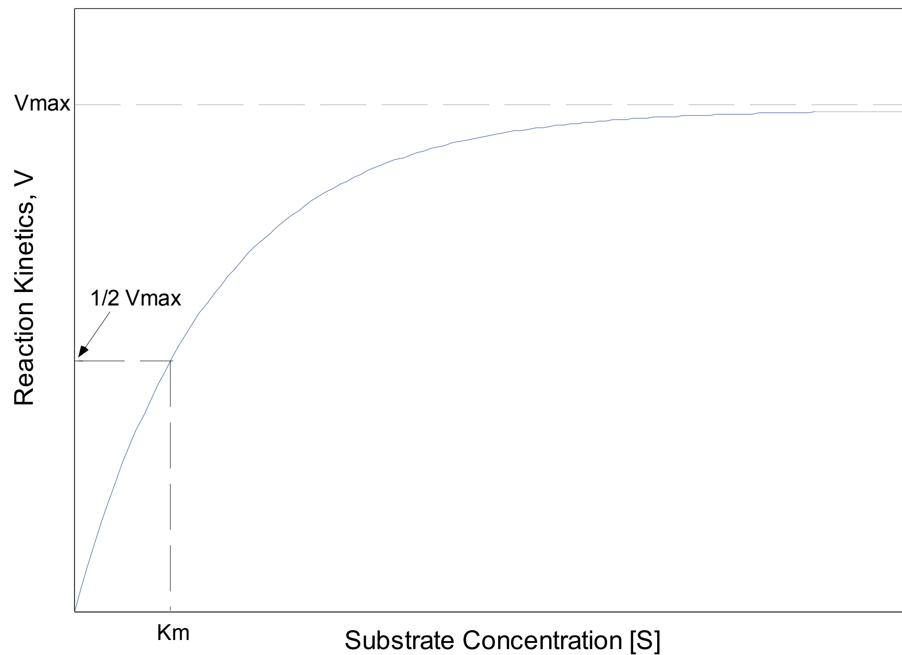


FIGURE 1.3. Michaelis-Menten Kinetics

1.3.3. Units for enzyme activity

The measured catalytic activity of enzymes can be expressed in terms of activity units present in a defined mass or volume of specimen. Enzyme activity is described as moles of substrate being converted to product per unit time. The SI unit is termed the *katal*, 1 katal is defined as 1 mole per second. However, in general enzyme assays katal is an excessively large unit. A more practical and commonly-used value is the international enzyme unit (U). 1 U is defined as 1 micromole per minute. 1 U corresponds to 16.67 nanokatals. It is noteworthy that activity is a measure of the quantity of active enzyme present and is thus dependent on experimental conditions, such as pH, temperature, type

of buffer, ionic strength, nature of substrate, concentration of activator/inhibitor, and other variables, which should be specified (Eisenthal and Danson 2002).

1.3.4. Factors affect enzyme kinetics

The Michaelis-Menten equation has shown that enzyme kinetics can be influenced by substrate concentration and product formation. However, there are other factors which can affect the catalytic activity of enzyme. The catalytic activity of enzyme depends on the integrity of its three-dimensional structure. Therefore, any disruption of its 3D structure would be accompanied with a loss of activity. Factors that can alter enzyme activity are temperature, pH, ionic strength, enzyme inhibitors and activators.

The three-dimensional structure of a protein is stabilized by hydrophobic/hydrophilic interactions, electrostatic forces, and hydrogen bonds. Changes in temperature, pH, and ionic strength in the preparation disturb the binding strength of electrostatic forces and hydrogen bonds. Therefore, the 3D structures of an enzyme as well as its complementary shape to the substrate are altered by the changing of electrostatic forces and hydrogen bonds. The deformation of catalytic site affects binding capability to substrate and ultimately leads to decay in catalytic activity, a process known as denaturation. If the process of denaturation is minimal, the catalytic activity can be recovered upon removal of the denaturing factors. However, prolonged or severe denaturing conditions would result in a permanent loss of enzyme activity (Bais and Panteghini 2006).

Theoretically, enzyme assay should be carried out at its optimal temperature and pH conditions because of two reasons. First, the slope near the apex of activity curve is minimal, so small variations in temperature or pH only cause negligible effects in enzyme activity. Secondly, because of the fastest enzyme kinetics at optimal pH and temperature, the yield of detectable signal is also the largest.

1.4. Enzyme Assays

In order to measure enzyme activity, it is necessary to transform substrate depletion or product generation into a detectable signal, such as property changes in absorbance, fluorescence, pH, enthalpy, conductivity, optical rotation or volume. Some enzyme-catalyzed reactions result in changes in the properties of the reactants which are relatively easy to measure directly, yet others do not. So indirect assays involve further treatments of reaction mixtures may be required.

While applying Michaelis-Menten kinetics, it is important to assure that one measures the initial, linear part of the progress curve of enzyme reaction (Figure 1.4) as the catalytic process may slow down because of the substrate depletion or reaching the reaction equilibrium. Owing to different sampling procedures, enzyme assays can be categorized to *continuous assay* and *discontinuous assay* (Eisenthal and Danson 2002). Continuous assay allows monitoring enzyme activity while the catalytic reaction is still in process. Discontinuous assay, on the other hand, requires termination of the ongoing reaction after a fixed interval and further treatment of the reaction mixture to separate a product or to produce a property change in the reactant. Given that the assay sensitivity is

sufficiently high and the procedure does not involve undesirable artifacts, continuous assays are usually preferred. Because they allow observation of progress curve, which largely simplifies the initial rates estimation and enables detection of irregular catalytic behaviors. However, discontinuous assays can become particularly attractive once the reliable reaction termination and product detection method has been established, because its capability of perform a large amount of incubations at the same time and therefore it is ideal for high-throughput, rapid-screening purposes.

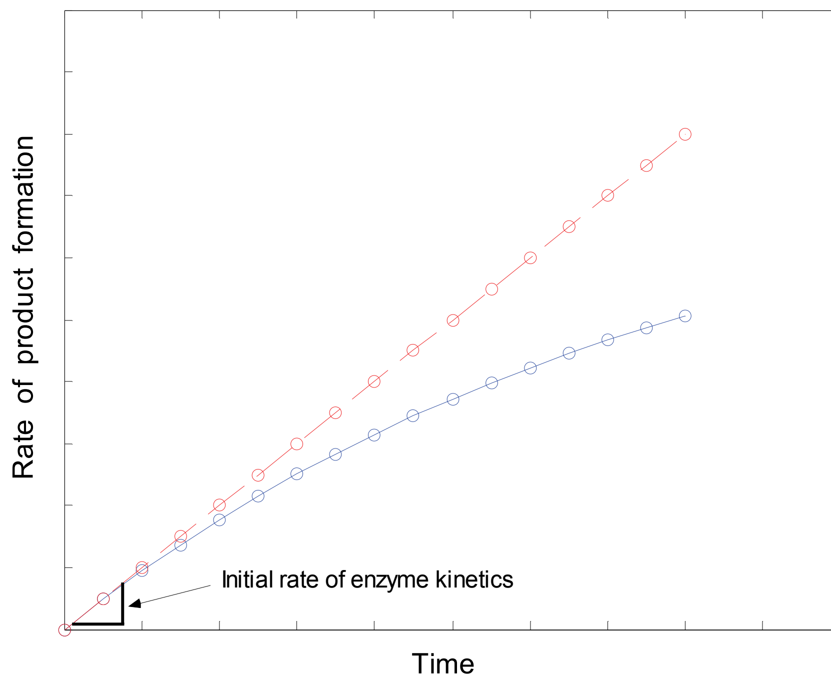


FIGURE 1.4. Initial rate measurement of enzyme kinetics. The red dash line shows the theoretical reaction kinetics to be measured. The actual enzyme reaction (blue solid line), however, may slow down over time due to the depletion of substrate or approaching the equilibrium of the reaction.

1.4.1. Continuous assay

Direct continuous assays such as pH-stat and spectrophotometric assays are always preferred due to their simplicity of estimation of progress curve and initial rates. Spectrophotometric assays are the most widely-used procedure in biomedical applications when the reaction involves an absorbance change. Because of the fact that common nucleotide coenzymes NADH and NADPH absorb UV light in their reduced forms but not in their oxidized forms, absorbance measurement at 340 nm can be readily applied for monitoring the enzyme kinetics. Fluorescent assay is also commonly utilized when a fluorescence change is involved with the reaction. This results in a considerable gain in the sensitivity and is particularly valuable when only small amounts of enzyme is present or very low product concentration. The pH-stat technique is more straightforward and convenient for measuring kinetics when the enzymatic reaction accompanied with pH change. By continuously adding acid or base to neutralize the pH shift, the kinetics of reaction can be readily obtained. Other continuous techniques such as calorimetric measurement (Todd and Gomez 2001) and gas detections such as O_2 or NO for assaying specific enzymes have also being reported (Kaku, Tanaka et al. 1994; Matsumoto, Takemata et al. 2002; Lei, Mulchandani et al. 2006).

1.4.2. Discontinuous assay

These assays involve stopping the reaction after a fixed period of time and treating the reaction mixture for product separation or modification of one of the substrates or products into the measurable form, which can then be analyzed. Liquid

chromatographic techniques are common discontinuous indirect assay for rapid reactant separation and quantification (Blank and Snyder 1991; Takahashi, Matsushita et al. 1993). Radiometric (Baudin, Beneteauburnat et al. 1990; Cole 1996) assay and chemiluminescence (Buxton, Edwards et al. 2000; Economou, Panoutsou et al. 2006) measurements are also frequently used. Enzyme substrates labeled with radioactive isotopes such as ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{125}I have been used in a variety of enzyme activity measurements and served as high-throughput screening assays in pharmaceutical industry. Firefly luciferase is well known for enabling bioluminescence emitting reaction and its gene is popularly used as a reporter gene. Luminescence enzyme assay offers high sensitivity and can be performed both *in vitro* and *in vivo*. Provided that oxygen is excessive, the formation or depletion of ATP can be determined by measuring the light emission in the presence of luciferase.

$$\text{Luciferin} + \text{ATP} + \text{O}_2 \xrightarrow{\textit{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{PP}_\text{i} + \text{CO}_2 + \textit{light}\ (h\nu)$$

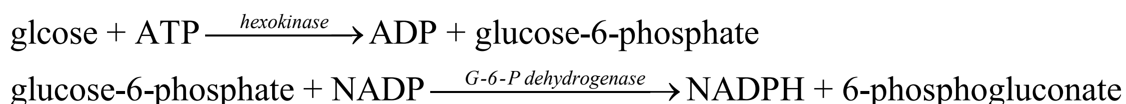
In addition to measure enzyme activity, discontinuous procedures can also serve as a tool to increase the sensitivity of detection. This can be illustrated by adding strong alkali into the mixture to convert NAD^+ or $NADP^+$ into highly fluorescent derivatives. These products fluoresce at 460 nm when excited at 370 nm with an intensity 10 folds higher than that given by $NAD(P)^+$/ NAD(P)H (Eisenthal and Danson 2002).

With all discontinuous assays, it is critical to ensure that the procedure used to terminate the reaction works instantaneously. If the volume of the assay mixture is relatively large, methods involving quick mixing with acid or alkali to alter the pH to a

value where the enzyme is inactive are usually more effective than methods involving transfer to an ice or boiling water bath.

1.4.3. Coupled assay

Only limited enzymatic reactions cause changes in the properties of the reactants that are relatively easy to measure directly and continuously. As to other reactions, it is necessary to use an indirect method that employs some further treatment of the reaction mixture. The most commonly used assays of this type engage the use of one or more consecutive enzyme reactions to catalyze a reaction of one of the products yielding detectable compound. Coupled assays often involve the reduction or oxidation common coenzymes, $NAD(P)^{+}$, because these processes can be readily determined spectrophotometrically or fluorometrically. The classical biochemical assay to measure glucose concentration, for instance, is to use hexokinase to catalyze glucose to gluocose-6-phosphate (Wright, Rainwater et al. 1971). Hexokinase activity can be obtained by adding glucose-6-phosphate dehydrogenase converting glucose-6-phosphate further to 6-phosphogluconolactone, the formation of NADPH can be used for detection.



$$\text{glcose} + \text{ATP} \xrightarrow{\textit{hexokinase}} \text{ADP} + \text{glucose-6-phosphate}$$
$$\text{glucose-6-phosphate} + \text{NADP} \xrightarrow{\textit{G-6-P dehydrogenase}} \text{NADPH} + \text{6-phosphogluconate}$$

For the coupled assay to be valid, it is critical to make sure that the subsequent reactions occur so rapidly that the first enzymatic reaction is always rate-limiting. Also it

is necessary that the reagent used for detection will not disturb other enzymes or components of the system.

Often times, one will find a variety of different assay methods available for the enzyme of interests. Hence the choice of assay simply depends on the convenience, availability of appropriate material and apparatus, as well as the purpose of study.

1.5. Principles of Developing Electrochemical pH-stat for Point-of-Care Testing

Optical enzyme assays are the most popular technique used in biomedical research and clinical laboratories. Even though considerable automation has been implemented to optical analyzers, it still requires clear sample for colorimetric measurements. Therefore, labor intensive and time consuming pre-treatment steps such as centrifugation, clotting factors' removal, and proper dilutions cannot be avoided. Therefore optical assays are not ideal for point-of-care analysis. The pH-stating technique we proposed, on the other hand, is a more general technique for monitoring kinetics in even untreated "raw" biological samples as the turbidity of sample will not have influence on the measurements.

A pH-stat utilizes continuous addition of acid (H^+) or base (OH^-) to maintain the sample at a pre-determined pH. Besides holding the pH, it can further be used to access the kinetics of the pH-changing reaction as it would be equal to the titrating rate of the acid/base reagent. In biomedical research, pH-stat is commonly utilized for measurements of enzyme activity (Brocklehurst 2002; Koditz and Ulbrich-Hofmann

2002; Spellman, McEvoy et al. 2003; Sias, Ferrato et al. 2004)., pH control in bioreactors (Fourest and Roux 1992; Hagedorn, Korlach et al. 1998; Kim, Lee et al. 2004; Tashiro, Takeda et al. 2004), and determination of acidity/alkalinity (Holm, Johansson et al. 1998; Joo, London et al. 1998).

1.5.1. Conventional pH-stat versus electrochemical pH-stat

Most pH-stat systems consist of a pH glass electrode immersed in the sample with its pH reading coupled with a feedback system to determine the deviation between actual and desired pH values. Based on the deviation, an acid or alkaline solution is continuously added so that the pH difference can be maintained at a minimum. Conventional pH-stat instruments use mechanical dosing burettes for volumetric titration and consequently, continuous sample dilution is an inherent problem. As the kinetics of biochemical reactions are critically dependent upon substrate/enzyme concentration, these systems are not suitable for sample size at micro-liter level because effects of dilution would not be negligible.

Electrochemical pH-stat utilizes water electrolysis to generate H^+/OH^- for the pH manipulation (Karcher and Pardue 1971; Adams, Betso et al. 1976; Hagedorn, Korlach et al. 1998). This technique could be very advantageous especially in biomedical applications for several reasons. (1) Coulometric acid/base addition by water electrolysis induces no appreciable volume change so that even samples of micro-liter level could be analyzed. (2) This approach eliminates the need for concentrated acid/base, so sample contamination can be avoided. (3) Dosing in an electrochemical pH-stat is delivered

amperometrically. Due to the fact that electrical current can be precisely controlled across a broad range, wide dynamic range of detection can be easily achieved.

In this work we developed a reagent-free pH-stat system using electrochemical titration and that it is suitable for enzymatic analyses in very small quantities of biological samples.

1.5.2. Electrochemical pH-stat on Rotating Sample System (RSS)

The principal platform that the majority of this work based on is named the Rotating Sample System (RSS). It is originated from the Biomedical Sensing Laboratory as a convective platform for different optical and electrochemical analyses in microliter-sized samples (Diefes, Hui et al. 1996; Xie and Gratzl 1996; Cserey and Gratzl 1997; Hui and Gratzl 1997; Shetty, Syed et al. 2005). The typical micro-liter sized sample (2-4 mm in diameter, 1-20 μL in volume) sits on top of a glass substrate with a working electrode (WE) and a liquid junction embedded in it (Figure 1.5). The sample droplet is surrounded by a hydrophobic ring made of silicon elastomer that keeps it in position. Due to the relatively large surface tension and capillary forces at this scale, an aqueous droplet assumes a nearly perfect hemispherical shape when a ring of proper inner diameter is used (4.2 mm for a 20 μL sample). Convection in the RSS is induced by two anti-parallel humidified air jets blown tangentially at the opposite sides of the droplet causing the sample to rotate about its axis. When used for electrochemical measurements, the RSS is comparable to the Rotating Disc Electrode (RDE) but is more suitable for analysis in smaller samples. Previous studies have demonstrated a number of useful applications of

the RSS such as diffusional microtitrations with both potentiometric and optical indication (Xie and Gratzl 1996; Hui and Gratzl 1997), trace metal detection using stripping voltametry (Cserey and Gratzl 1997; Shetty, Syed et al. 2005), electrochemical pH-stating (Kao, Hsu et al. 2008; Kao and Gratzl 2009), and application of spatially averaging electrodes (Sheth, Diefes et al. 2009). It is worthwhile to mention that in this work, we also explored a new area that uses the RSS to detect changes in surface properties. This technique can be applied to determine critical micelle concentration (CMC) of surfactants as described in the chapter 3 (Kao, Shetty et al. 2008).

The reagent-free micro pH-stat is based on RSS platform with a micro pH electrode added on top of the sample. Titrant of acid or base is produced by water electrolysis by a 250 μm platinum working electrode embedded in the substrate. Effective convection homogenizes the pH gradient within the sample and assures correct pH feedback for the controller. The unique feature of RSS eliminates the major difficulty of generating convection in droplets. As water electrolysis induces virtually no volume change, pH-stating *in vitro* enzyme activities in very small samples such as 1-20 μL range become feasible. Results of cholinesterase assays in both buffer and serum using proposed electrochemical pH-stat have shown to be comparable with standard optical techniques ($r^2 \geq 0.97$). We expect that this novel technique has great potential to become a miniaturized analyzer for point-of-care diagnostics.

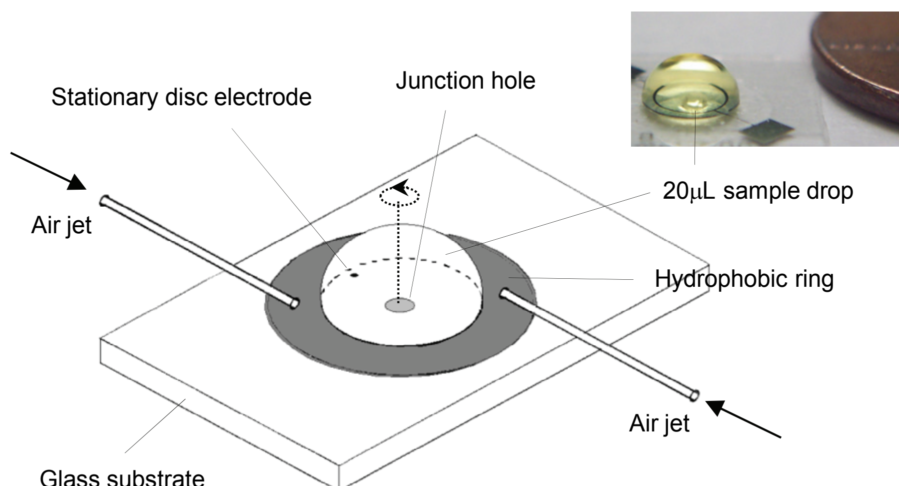


FIGURE 1.5. Schematic diagram of the Rotating Sample System. The position of the Pt mini-disk working electrode (WE) to the axis of rotation can be variable. A liquid junction is made of a hole (1 mm in diameter) filled with agar gel connecting the sample to an underlying compartment where reference electrode (RE) and counter electrode (CE) are placed (Shetty 2005).

1.5.3. Limitations

Even though many advantages have been offered, there are several limitations for using the electrochemical pH-stat system:

(1) Aqueous sample is required for this approach. Electrochemical pH-stat employs water-splitting to produce acid and base so titration cannot occur without aqueous solution. Therefore non-aqueous samples, e.g., organic solvents and pure lipid/oil cannot be analyzed.

(2) Current efficiency can be affected in the case of some electroactive compounds present in the sample. Side electrochemical reactions such as chloride oxidation reduce current efficiency so removal of electroactive molecules, or coating isolation membrane on the surface of the electrode, or performing calibration for current efficiency prior to the measurement may be required in those cases.

Nevertheless, it is worthwhile to note that oxygen reduction generates the same amount of hydroxyl ions as water electrolysis so presence of oxygen in the solution does not affect current efficiency.

(3) To be analyzed by pH-stat assay, the enzymatic reaction needs to involve pH shift (ie, generating either H^+ or OH^- as a side product. However, if the enzyme of interest or the reaction itself does not produce pH change, coupled enzymes reactions could be applied to achieve an eventual pH shift.

1.6. Conclusion

With the scientific advances today in identifying disease markers, blood analysis has become the primary measure in diagnosis of many physiological and pathological conditions. Point-of-care testing demonstrates the potential of reducing turnaround time, labor, and laboratory cost substantially and thus promotes better medical outcome. We studied an electrochemical micro pH-stat as a universal platform for rapid detection of enzyme activity and substrate concentration. Because of the advantages of operation in untreated biological samples, precise amperometric measurement, the ease of being miniaturized, this system is particularly suited for point-of-care testing. Table 1.1 lists the

enzymes of clinical significance that can be analyzed by pH-stat technique. We chose cholinesterase as a model enzyme to validate the system. The results in buffer and serum are in good agreement with standard techniques.

We also explored a new concept that utilizes the RSS as a tool to monitor the variations of surface tension of samples. This is made possible by the fact that convection is afforded *via* the air/liquid mechanical coupling. Therefore, changes in surface tension affect the velocity of the flow and hence can be detected by using hydrodynamic electrochemistry of an electroactive probe in the sample. This technique can be applied for the determination of CMC, study of protein-protein surface interactions, and assays for blood coagulation.

In addition to clinical and biomedical use, this system may also find applications in environmental, agricultural, and food industries.

TABLE 1.1. Enzymes of clinical importance that can be analyzed by pH-stating

Enzyme name	Principal Source of enzyme in blood	Clinical Significance	Available POCT
Creatine kinase	Skeletal muscle, heart	Muscle diseases, myocardial infarction	i-STAT (amperometry) Piccolo (optical)
Cholinesterase	Liver	Insecticide poisoning, suxamethonium sensitivity, parenchymal diseases	EQM Test-Mate (optical)
Lipase	Pancreas	Pancreatic diseases	N/A
Lactate dehydrogenase	Heart, liver, skeletal muscle, erythrocytes, platelets, lymph nodes	Hemolysis, hepatic parenchymal diseases, myocardial infarction	Piccolo (optical)
Aspartate aminotransferase	Liver, skeletal muscle, heart, erythrocytes	Hepatic parenchymal disease, muscle disease	Reflotron (optical) Piccolo (optical)
Alanine aminotransferase	Liver, skeletal muscle	Hepatic parenchymal disease	Reflotron (optical) Piccolo (optical)
Urease	Plants, intestinal microorganisms	Urinary tract pathogens	Rapid strips
Glutamate dehydrogenase	Liver	Hepatic parenchymal disease	N/A
Amylase	Salivary glands, pancreas	Pancreatic disease	Reflotron (optical) Piccolo (optical)

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Chapter 2

Reagentless pH-stat for Microliter Fluid Specimens

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This manuscript is a proof-of-concept of the electrochemical micro pH-stat and it demonstrates pH-stating in a 20 µL sample for the first time. Hence the work described in this chapter has built the foundation of this research.

2.1. Abstract

pH-stating is a common technique for monitoring kinetics of various (bio)chemical reactions that involve generation of hydrogen or hydroxyl ions. In this work we describe a reagentless electrochemical micro pH-stat where the titrant of acid or base is produced by water electrolysis on the Rotating Sample System (RSS) platform. RSS has originated from the authors' laboratory as a convective platform to support different analytical techniques in microliter sized samples. As water electrolysis induces no volume change and the current that generates the reagent can be precisely measured even at low levels, very small samples in the microliter range become accessible for pH-stating: a reduction of more than an order of magnitude in specimen size relative to the most sensitive conventional methods. Nearly 100% current efficiency has been achieved with this system using a 250 µm Pt mini-disc working electrode for electrolysis. The developed micro pH-stat has been validated by the determination of the activity of erythrocyte acetylcholinesterase (AChE) as a function of substrate concentration and pH. The optimal pH and activity profile obtained are in good agreement with those determined with standard techniques. The micro pH-stat has the potential for applications for enzyme assays, reagentless pH control, acidity/alkalinity, and buffer capacity measurements in very small samples of biomedical and environmental origin.

2.2. Introduction

We report here on a reagentless pH-stat system that is suitable for precise pH-stating of microliter samples. In a pH-stat acid or base is added to the sample at a rate that keeps the pH at a predetermined value despite of an ongoing reaction that would tend to steadily shift it. The rate of reagent addition necessary to maintain the preset pH is equal to the rate of reaction that is to be determined.

pH-stating is important in pH control in bioreactors(Fourest and Roux 1992; Hagedorn, Korlach et al. 1998; Kim, Lee et al. 2004; Tashiro, Takeda et al. 2004), determining acidity/alkalinity (Holm, Johansson et al. 1998; Joo, London et al. 1998), and monitoring enzyme activity (Brocklehurst 2002; Koditz and Ulbrich-Hofmann 2002; Spellman, McEvoy et al. 2003; Sias, Ferrato et al. 2004). Accurate pH control is generally required for biological preparations such as cell culture (Hagedorn, Korlach et al. 1998; Kim, Lee et al. 2004), fermentation (Fourest and Roux 1992; Tashiro, Takeda et al. 2004), and enzyme analysis (Brocklehurst 2002; Koditz and Ulbrich-Hofmann 2002; Spellman, McEvoy et al. 2003; Sias, Ferrato et al. 2004). Since the activity of enzymes is critically dependent on pH, pH-stating is a convenient way to maintain a constant pH and at the same time monitor the kinetics of enzymatic reactions that generate protons or hydroxyl ions (Brocklehurst 2002; Koditz and Ulbrich-Hofmann 2002; Spellman, McEvoy et al. 2003; Sias, Ferrato et al. 2004). Besides, the pH-stat can also be used for other applications such as quantification of mucosal acid/alkaline secretion for studying gastrointestinal regulation (Holm, Johansson et al. 1998; Joo, London et al. 1998) and determination of buffer capacity (Claessens, Behrends et al. 2004).

Spectrophotometric assays are the most popular schemes for measuring enzyme activities when variations of optical properties arise with the chemical transformation of substrate to product. The corresponding variations in light absorbance or fluorescence are detected and correlated to reaction kinetics (John 2002). The pH-stat method complements spectrophotometric methods, applicable to monitor enzymatic reaction where changes in proton binding sites occur during the catalytic process rather than changes in chromophoric character. This allows the pH-stat to measure turbid samples such as blood or cellular extracts and study enzyme reactions which do not intrinsically lead to color changes (Brocklehurst 2002).

Conventional pH-stats employ fine mechanical dosing burettes that deliver the required titrant into the sample continuously so as to maintain its pH. Due to continuous volumetric reagent addition sample dilution occurs. If the volume of available sample is abundant, using a sample size in the milliliter range and concentrated acid/base reagents can make relative volume changes negligible. However, sample dilution would become significant in smaller specimens. As the kinetics of biochemical reactions are critically dependent upon substrate concentration, dilution needs to be corrected for in the data analysis (Tsibanov, Loginova et al. 1982).

Electrochemical pH-stating (Karcher and Pardue 1971; Adams, Betso et al. 1976; Hagedorn, Korlach et al. 1998) could be more attractive than conventional pH-stating especially in biomedical applications for several reasons. Coulometric acid/base addition by water electrolysis induces no appreciable volume change so that even samples of very small volume could be analyzed. This approach also eliminates the need for additional reagents and thus, prevents specimen contamination. Dosing in an electrochemical pH-

stat is accomplished by electrical current injection which can be accurately controlled even at very low levels. With proper current control the electrochemical scheme can also be made calibration free by virtue of Faraday's law.

A few coulometric pH-stat systems for measurement of enzyme activity (Einsel, Trurnit et al. 1956; Karcher and Pardue 1971; Adams, Betso et al. 1976) have been reported decades ago. In 1971, Karcher and Pardue have applied a pulsed electrolysis current source to produce H^+/OH^- titrant in a photometer cell while monitoring the pH with a pH indicator (Karcher and Pardue 1971). The reason for using colorimetric pH detection was to avoid interference between the current-generating circuit and the pH-sensing system that has been observed with potentiometric pH detection when both have a common ground (Adams, Betso et al. 1976). Adams *et al*. constructed an entirely electrochemical pH-stat with an isolation amplifier to eliminate this cross-talk. Both reported systems required sample volumes in the range of 3.5-50 mL, limited by the need for effective convectional mixing which is difficult to achieve in very small specimens. Thorough mixing is important in pH-stating to ensure that the added acid/base distributes instantaneously and uniformly in the sample that is essential for efficient feedback control.

We report here on a reagent-free micro pH-stat based on the concepts of the Rotating Sample System (RSS) which is a general convection platform developed in the authors' laboratory for microliter-sized samples (Diefes, Hui et al. 1996; Xie and Gratzl 1996; Cserey and Gratzl 1997; Hui and Gratzl 1997; Shetty, Syed et al. 2005). Convection in the RSS is induced by rotating a semi-spherical aqueous sample drop on top of a stationary substrate such as glass with a working electrode (WE) and a liquid

junction embedded in it. The sample is surrounded by a hydrophobic ring that keeps it in place. Due to surface tension and capillary forces, an aqueous droplet assumes a nearly perfect hemispherical shape when a ring of proper diameter is used. Convection is induced by two anti-parallel humidified air jets blown tangentially at the opposite sides of the droplet causing the sample to rotate about its axis. When used for electrochemical measurements, the RSS was found to be equivalent to the Rotating Disc Electrode (RDE) but suitable for measurements in very small specimens. Convection within a 20 μL sample has been shown to achieve a diffusion layer thickness in the order of 10 μm (Shetty, Syed et al. 2005) which is equivalent to a disc electrode rotated about 3000 rpm. Previous studies have demonstrated a number of useful applications of the RSS such as diffusional microtitrations with both potentiometric and optical indication (Xie and Gratzl 1996; Hui and Gratzl 1997), and trace metal detection using stripping voltametry (Cserey and Gratzl 1997; Shetty, Syed et al. 2005).

The reagentless pH-stat system reported in this work is built on the RSS platform with an added combination pH micro electrode on top of the fluid specimen (Figure 2.1). Acid or base titrant is produced by electrolysis at the Pt working electrode at which essentially 100% current efficiency is achieved in inert buffer solution. This setup effectively operates as a galvanostat with an output range of 0.1-100 μA, which provides a broader range of analysis when compared to spectrophotometry.

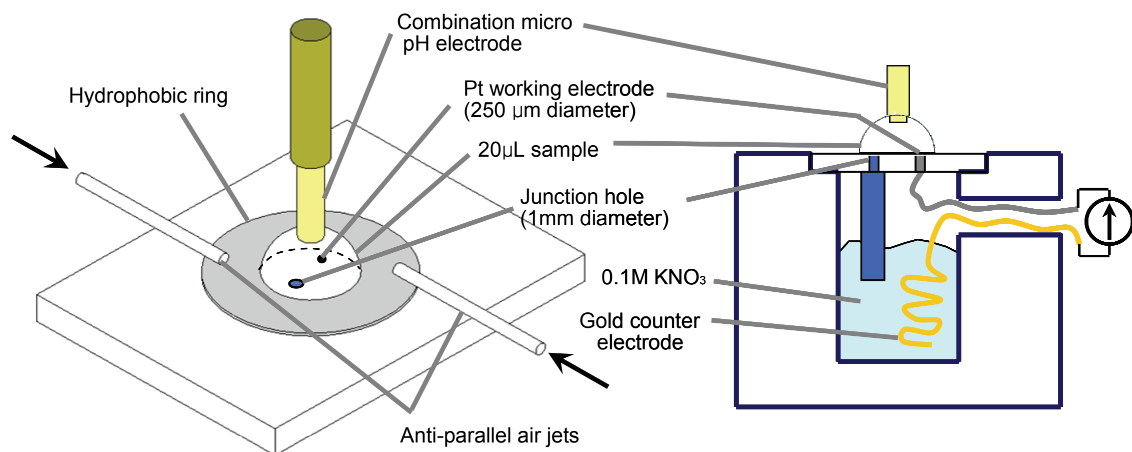

FIGURE 2.1. Schematic diagram of the reagentless micro pH-stat based on the Rotating Sample System platform (RSS). The liquid junction connecting the sample to the gold counter electrode in the underlying compartment is filled with 1% w/w agar gel in the same supporting electrolyte solution, 0.1 M potassium nitrate, as that of the lower compartment.

We have determined the activity of acetylcholinesterase (AChE) in buffers in this work for optimizing and validating the reagentless micro-pH-stat system. Inhibition of AChE activity is generally used as a biomarker of neurotoxicity from exposure to organophosphate compounds in industrial chemicals and insecticides (Worek, Mast et al. 1999; Panteghini, Bais et al. 2006). AChE breaks down its substrate, acetylcholine, to choline and acetic acid. AChE assays in biochemical studies are generally performed by spectrophotometry using Ellman's method (Ellman, Courtney et al. 1961) in pH 8 buffer. Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), however, can react with sulfhydrl groups of cell or tissue extracts and interfere with the assay (Ellman 1959).

Therefore a blank sample consisting of the extract, buffer, and DTNB may be required to correct for the potential release of thiol material and the absorbance of the other substances in the preparation (Ellman, Courtney et al. 1961). The pH-stat technique (Karcher and Pardue 1971; Garcialopez and Monteoliva 1988) is an alternative and low-cost method for determining AChE activity. We have demonstrated AChE assays in 20 μL sample volumes assessing enzyme kinetics at different substrate concentrations and set pH values without the need for exogenous reagents. The optimal pH and activity profiles have shown good agreement with results obtained with standard spectrophotometry techniques.

2.3. Experimental

2.3.1. Apparatus

Micro pH-stat design: A RSS platform (Figure 2.1) operating as a galvanostat is used for electrochemical acid/base addition and reagent mixing with a 250 μm diameter Pt mini-disc working electrode (Alfa Aesar, Ward Hill, MA, USA) and a liquid junction embedded. Fabrication procedures are described in previous work (Shetty, Syed et al. 2005; Kao, Hsu et al. 2006). A micro pH electrode (MI-4154 Microelectrodes, Inc., Bedford, NH, USA) with 1 mm tip diameter is connected to a Fisher AR-15 digital pH meter to monitor pH of the sample. pH values are acquired at the rate of 1 Hz via the RS-232 port of the computer. Between the pH meter and the computer, an RS-232 optical isolator (Model 9POP4, B&B Electronics Inc., Ottawa, IL, USA) was adopted to avoid cross-talk between current generation and pH detection. The controller program

(LabVIEW, National Instruments Corp., Austin, TX, USA) serves as both user interface and PID control algorithm. The output signal from the controller program is calculated by comparing the error with respect to the pre-set pH value using optimized PID parameters, and sent to the current generation unit.

Current source for electrolysis: The current generating unit consists of a DAQ Pad (6020E, National Instruments) and a voltage-to-current (VTC) converter circuit. The output signal from the LabVIEW program is sent and amplified through the DAQ Pad via a USB port; the amplified voltage output is then converted into the desired current by the VTC circuit. The current output could be adjusted from ±0.1 to ±100 μA. The load independent VTC circuit is capable of overcoming sample impedance up to 100 kΩ, which is about 20 fold higher than the total impedance of the system (~5 kΩ). A 10 kΩ potentiometer is employed as a common null adjustment to account for eventual baseline shift in the OP-AMP circuit. Thus, the background noise is at least two orders of magnitude less than current output. An independent current meter (Keithley Instruments Inc., Cleveland, OH, USA) is used for monitoring and verifying the actual current passing through the sample.

Minimizing influx of atmospheric CO_2: Due to the large surface-to-volume ratio of a 20 μL sample, the pH of the solution could get noticeably acidified by influx of CO_2 from air. In order to minimize this effect, a closed acrylic chamber with a DI water reservoir surrounding the RSS cell was fabricated to keep the cell in a humidified and isolated environment. A small hole was made to equilibrate air pressure inside the chamber with the outside atmosphere. The chamber also provides for positioning of the

combination pH electrode and the two anti-parallel air nozzles. The air used for sample rotation flows through porous air stone to produce fine air bubbles in saturated $Ca(OH)_2$ providing a large total surface area for CO_2 absorption thereby minimizing exposure of the sample to CO_2.

2.3.2. Materials

All chemicals were from Sigma (Sigma Chemical Co., St. Louis, MO, USA) and Fisher (Fisher Scientific, Pittsburgh, PA, USA). All aqueous solutions and subsequent dilutions were prepared by using 18.2 MΩ-cm deionized water from Millipore Milli-QUV plus (Billerica, MA, USA).

pH-stat cell: The substrate of the pH-stat cell is made from a microscope slide. 1% w/w Type I agarose was prepared in 0.1 M KNO_3 solution and filled into the junction hole embedded in the glass substrate. A hydrophobic ring of 4.2 mm inner diameter for confining the hemispherical sample (Figure 2.1) is made of silicon elastomer (DOW Corning, Midland, MI, USA) coated onto the substrate.

AChE assay: Solutions were prepared freshly before experiments. For pH-stat assay 2.5 U/mL of acetylcholinesterase (AChE) from bovine erythrocytes prepared in stock solution of 0.1 M NaCl was used. The substrate acetylthiocholine iodide (ATCh) was prepared in 0.1 M NaCl and adjusted to the desired pH by NaOH or HCl. 0.15% Intralipid (Baxter Healthcare Corp., Deerfield, IL) was added to the enzyme solution

before the pH-stat assay to break up the protein layer adsorbed onto the sample-air interface, thus ensuring efficient coupling of the air jets for rotation.

All solutions for the colorimetric AChE assay were prepared in 0.1 M PBS with the pH adjusted to the desired value. 1.5 mM DTNB (Ellman's reagent) is used as color-changing reagent.

2.3.3. Procedures

General Procedures for pH-stating: Prior to each measurement a 20 μL droplet of 0.1 M KNO_3 is placed atop the RSS cell and a 0 to -1 V pre-conditioning CV scan at 0.1 V/s scan rate is applied 10 times to clean and stabilize the WE surface. The droplet is then removed by a transfer pipette and replaced with a new sample. The standard pH-stating process is applied afterwards.

Determination of current efficiency: As the reagentless pH-stat system makes use of electrolysis to produce the required acid or base, the actual amount of H^+/OH^- generated by a given current needs to be determined quantitatively. Droplets of 0.1 M KNO_3 with little or no phosphate buffer depending on the current range is used for this purpose. A pH change of typically 0.5-1 pH unit is induced by adding known amounts of KOH or HNO_3 of 4-9 μL and concentrations from 0.02-8 mM to the droplet to make a final volume of 20 μL.

A constant current is subsequently delivered to the drop generating H^+/OH^- to bring the pH back to its initial value. When the original pH is reached the current is

stopped and the duration of current injection is recorded. Current efficiency in this work is defined as moles of KOH/HNO_3 added to the sample divided by the added charge necessary to restore the initial pH x 100%.

Determination of enzyme activity by pH-stating: The activity of AChE as a function of substrate concentration in the 0.1-20 mM range is measured at room temperature. 15 μL substrate solution is placed on top of the RSS cell. Once the pH reading is stabilized at 8 the PID controller is turned on. 5 μL of 20-100 folds diluted AChE solution (initial concentration 2.5 U/mL) is then added to the substrate solution. The enzymatic reaction generates protons that are compensated by pH-stating. The duration of each experiment lasts at least for one minute. By averaging the current over the period of pH-stating and applying Faraday's law, the enzyme activity at the given substrate concentration is obtained.

The determination of AChE activity versus pH variation follows the same procedure except that 2.5 mM substrate is used for all measurements and the pH is varied from 6.5 to 8.5.

Control of AChE assay: AChE assay using Ellman's procedure has been adapted as control. A 0.5 mL mixture of 0.1 mL DTNB (final concentration 0.3 mM), 0.1 mL ATCh (final concentration 0.1-20 mM), and 0.3 mL diluted AChE (initial concentration 2.5 U/mL) is analyzed using a spectrophotometer (Gilford Response) at room temperature. 10 readings are taken at 15-second intervals at 412 nm and the reaction rate is calculated. The extinction coefficient used for DTNB is 13600 $M^{-1}cm^{-1}$ (Ellman, Courtney et al. 1961).

2.4. Results and Discussion

2.4.1. System characteristics

The challenges of the proposed electrochemical micro pH-stat include potential interference by common ground in the electrical system, IR drop in the wet electrochemical system, difficulty of homogenization of microliter-sized samples, and influx of atmospheric CO_2. As mentioned above, cross-talk between current generation and pH sensing may be present in an electrochemical pH-stat system. When current injection is active, in case of using a common ground for both the current injection output and the sensing circuit a voltage shift generated by the former could propagate to, and affect the reference potential of the pH meter resulting in erratic pH reading. An optical isolator was used in this work to intercept this voltage propagation between the current injecting circuitry and the pH meter so that there is no observable voltage shift during current injection.

In addition to potential electrical interference that can occur in the electrical system, there may also be electrochemical interference in the wet electrochemical system between current output and pH sensing. This problem originates from the current injected for electrolysis passing through the RSS cell. Due to sample and junction impedances, an IR drop between the pH sensitive electrode and the Ag|AgCl reference may develop causing the pH measurement to be biased. This can be mitigated in two ways: (1) Using a RSS cell with two junctions to separate the counter electrode for current injection and the reference electrode for pH sensing. (2) Using a combination pH electrode to minimize the IR drop between the pH sensing electrode and its reference. The first method has shown

promising results in eliminating uncertainties due to IR drop. However, the design of an RSS cell with two junctions substantially increases the complexity of cell fabrication. In addition, the difficulty of having to place two junctions and one Pt working electrode in a relatively small region (4.2 mm diameter circular area) can lead to poor electrical isolation. We found that some level of leakage current between the junctions and the Pt electrode was hard to avoid. To address this problem, a micro combination pH electrode instead of a two-junction design was adopted. Due to the very short distance (~0.2 mm) between the pH-sensing membrane and the junction of the micro pH electrode, the IR drop caused current injection was found to be negligible.

Effective convection is necessary to ensure continuous homogenization of the sample and minimal delay between current injection and pH reading for feedback control. Two superimposed flow patterns could be identified from theory of the RSS derived using the Navier-Stoke equations and Prandtl's boundary-layer theory (Shetty, Syed et al. 2005). The primary flow in the droplet is rotation about its vertical axis. In addition, a secondary flow in vertical planes occurs in the droplet bulk. These interacting flow patterns create a hydrodynamic system that is equivalent to a Rotating Disc Electrode (RDE) and ensures effective mixing as has been demonstrated in previous studies (Cserey and Gratzl 1997; Hui and Gratzl 1997; Shetty, Syed et al. 2005).

The pH of a 20 μL droplet with its high surface to volume ratio can be affected by evaporation, and influx of CO_2 from the atmosphere. Keeping the sample in a closed chamber and humidifying the air flow in saturated $Ca(OH)_2$ buffer have substantially reduced the effects of both these potential problems. As a consequence, background pH drift at pH 8, which is the desired pH value specific for the AChE assay, was reduced

from 0.8 to less than 0.03 during 4 minutes of sample rotation. It is noted that pH of the $Ca(OH)_2$ buffer needs to be properly adjusted, or it needs to be replaced with other suitable buffer when the desired pH value is lower than 8.

2.4.2. Current efficiency

Measured current efficiency values over the whole output range of the system are shown in Table 2.1. Cyclic Voltammetry (CV) has been performed to confirm that no redox reactions occur in any of the samples (buffer, substrate, and enzyme) other than O_2 reduction and water-splitting. It is noteworthy that eventual O_2 reduction yields the same amount of OH^- at the Pt cathode per electron as water electrolysis, so the overall current efficiency will not be affected by the presence of dissolved oxygen.

Current applied in the cathodic direction generates OH^- ions with nearly 100% (99.9 - 100.8%) efficiency even at low current levels. This indicates that current density at the Pt mini-disc electrode is high enough so that virtually all injected charge turns into OH^-. However, current efficiency in the anodic direction is slightly higher than the nominal 100%. This may result from acid shift in weakly alkaline solutions due to CO_2 influx from air. Consequently, fewer protons need to be generated for titration that leads to over-estimation of current efficiency.

Identical protocols have been performed in substrate (ATCh), enzyme (AChE), and mixed solutions of substrate, enzyme, and product as controls. These tests were done only in the cathodic direction since OH^- is needed for pH-stating of the AChE reaction.

No observable changes were found with respect to current efficiency obtained in KNO_3 solution.

TABLE 2.1. Current efficiency of the micro pH-stat in KNO_3 buffer

anodic current (H^+ generation)*			cathodic current (OH^- generation)		
I (μA)	eff. (%)	std(%)	I (μA)	eff. (%)	std(%)
-	-	-	-0.1	99.9	0.4
-	-	-	-1	100.0	0.3
10	104.3	1.4	-10	100.8	1.0
20	101.3	1.1	-20	100.1	0.3
40	104.5	0.8	-40	99.9	0.8
60	107.0	1.6	-60	99.9	0.9
80	102.7	2.6	-80	100.3	0.2
100	104.3	5.2	-100	100.4	0.7

*The concentration of KOH for measuring anodic current efficiency at 1 and 0.1 μA needs to be so diluted (0.2-0.02 mM) that CO_2 influx from air is too significant for the data to be used for measurement.

2.4.3. Determination of AChE activity

We demonstrated the applicability of using the proposed micro pH-stat for enzyme assays by the determination of AChE activity. The control system with optimized PID parameters was capable of keeping the sample pH stably within 0.05 pH unit of the desired value. Given the essentially 100% current efficiency, the total charge delivered

for pH-stating would correspond to the absolute amount of protons generated by the enzymatic reaction.

A complete analysis takes about 2-4 mins including placing the sample, pH stabilization, adding the enzyme, and pH-stating. AChE activities obtained with pH-stating, and comparison with results of the standard Ellman's method are shown in Figure 2.2 and 2.3. Results of the two different schemes are close to each other (correlation coefficients are 0.99 and 0.97, respectively). Catalytic activity of AChE is known to be inhibited by its substrate upon binding to the peripheral site (Szegletes, Mallender et al. 1999). Our results indicate a maximum rate occurring at 1.5 mM substrate concentration, which is in good agreement with reported data (Karcher and Pardue 1971). Variation in AChE activity with respect to pH (Figure 2.3) shows that the optimal pH is near pH 7.6 which agrees well the reported value (Karcher and Pardue 1971). The average standard deviation ($n \geq 3$) for the pH-stat results and for the control assays are 3.7% and 5.4%, respectively.

The enzyme solution used in the colorimetric assay was diluted 100 times to reduce turbidity and to accommodate the limited linear detection range of the spectrophotometer. In the pH-stat assay no dilution was necessary since turbidity does not affect the measurement and the detection range is defined by the current output range which can be easily adjusted. However, dilution (20-100 folds) of enzyme samples was still applied in the pH-stat assay to ensure a constant rate of enzyme kinetics despite the low substrate concentrations used (0.1-20 mM). The color-changing reagent DTNB is obviously not needed. The current output range of the micro pH-stat system, 0.1-100 μA, corresponds to 3.1 U/L-3.1 U/mL which is a practical range for enzyme assays.

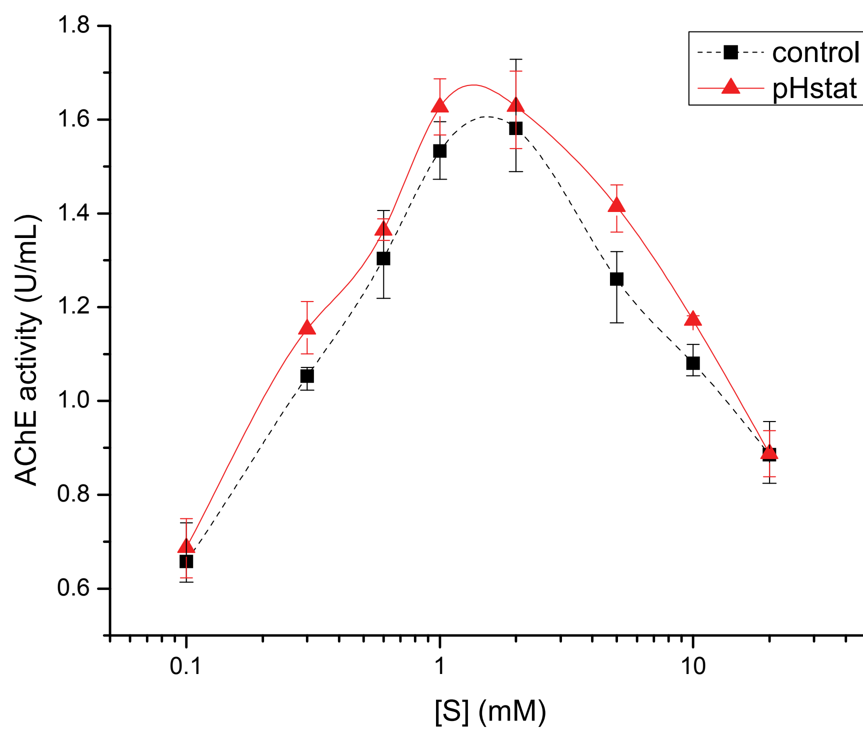

FIGURE 2.2. Substrate dependence of AChE activity determined with the micro pH-stat (▲, in red) and with spectrophotometry as control (■). The maximum activity occurs at ~1.5 mM substrate concentration due to inhibition by its own substrate at high level. Coefficient of correlation between the data sets obtained with the two techniques is 0.99.

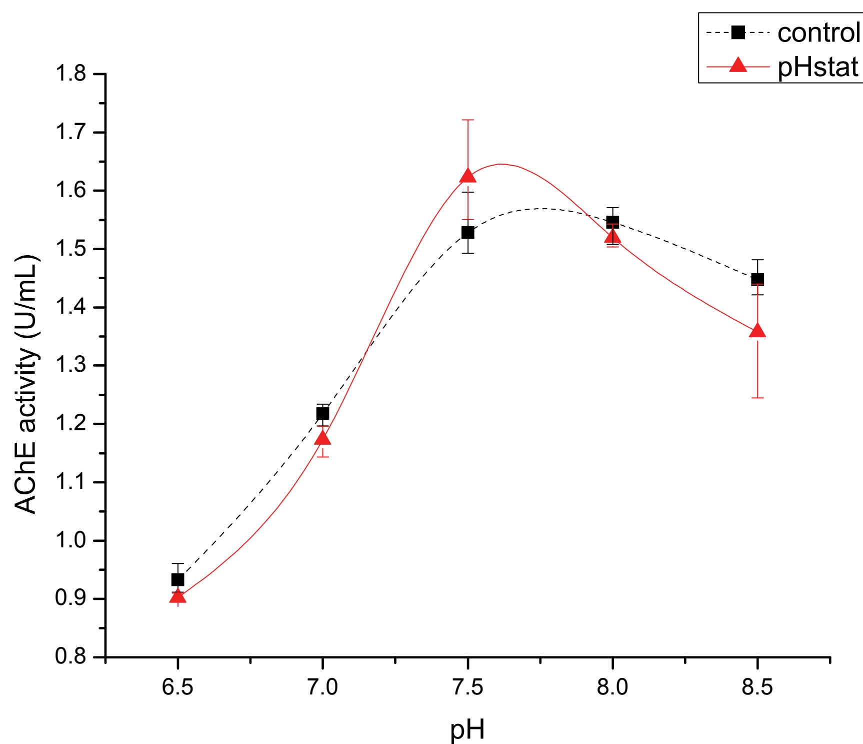

FIGURE 2.3. pH dependence of AChE activity. The optimal pH of AChE is at ~7.6 as observed with both the pH-stat assay (▲, in red) and the optical control (■). The coefficient of correlation between the data sets obtained with the two techniques is 0.97.

2.5. Conclusions

Feasibility of a reagentless micro pH-stat has been demonstrated in this work. In contrast to conventional pH-stats, no complex mechanical delivery mechanisms or strong acid/base reagents are needed. Because of non-volumetric acid/base addition and the use of the RSS platform for mixing, precise measurements can be achieved in microliter samples. This is particularly beneficial for biological assays such as samples from small laboratory animals (Davidson, Doran et al. 1994; Abdel-Majid, Leong et al. 1998; Chanas,

Jiang et al. 2002). The pH-stat system is capable of measuring enzyme activities as low as 60 μU in 20 μL specimens. Results obtained on the AChE model system show good agreement with the standard optical assay.

Owing to the large surface area relative to the small sample volume, absorption of CO_2 and diffusional pH titration (Hui and Gratzl 1997) from the liquid junction may cause pH drift, especially in non-buffered samples. Therefore, precautions must be taken to minimize these potential interferences.

Taking advantage of essentially 100% current efficiency of OH^- generation, this system is suitable for monitoring proton-generating enzyme reactions without the need for calibration. It should be noted, however, that the use of a platinum anode for H^+ injection such as when analyzing urease activity requires anodic potentials around 1.2 V which can oxidize chloride and some biomolecules such as ascorbate and uric acid. Therefore, current efficiency may be lower than 100% in such samples and calibration for proton generation may be required.

2.6. Acknowledgements

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Chapter 3

Determination of Critical Micelle Concentration with the Rotating Sample System

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This manuscript is an application of the Rotating Sample System (RSS) that does not involve pH-stating. Here we explore a new area, probing of the properties in both the solution bulk and the air/liquid interface in a microliter-sized sample.

3.1. Abstract

A novel experimental approach using the Rotating Sample System (RSS) is proposed here for the determination of critical micelle concentration (CMC) of surfactants. The RSS has been conceived in our laboratory as a convection platform for physicochemical studies and analyses in microliter sized sample drops. The scheme allows for vigorous rotation of the drop despite its small size through efficient air-liquid mechanical coupling. Thus, changes in surface properties of aqueous samples result in corresponding modulation of the hydrodynamic performance of the RSS, which can be utilized to investigate interfacial phenomena. In this work, we demonstrate that the RSS can be used to study the effects of surfactants on the surface and bulk of very small samples with hydrodynamic electrochemistry. Potassium ferrocyanide is employed here with cyclic voltammetry to probe the air-water interface of solutions containing Triton X-100. The CMC of this surfactant determined using this approach is 140 ppm which agrees well with reported values obtained with conventional methods in much larger samples. The results also demonstrate that besides the CMC, variations in bulk rheological properties can also be investigated in very small specimens using the RSS with simple methodology.

3.2. Introduction

The Rotating Sample System (RSS) is a unique convection platform conceived in our laboratory for analyses and physicochemical studies in very small drops of different specimens (Xie and Gratzl 1996; Cserey and Gratzl 1997; Hui and Gratzl 1997; Shetty, Syed et al. 2005; Kao, Hsu et al. 2008). Here, we propose the application of the RSS for the determination of critical micelle concentration (CMC) of surfactants in microliter sized samples.

The study of interfaces is of great interest to researchers across different areas of science. Examination of a surface in the presence of surface-active molecules such as proteins, lipids, and surfactants provides insight into the physicochemical properties of the surface and the bulk of solutions. Protein-lipid interactions at aqueous-air interfaces play a crucial role in the stability of these systems (Poole 1989). Pulmonary surfactants are used to prevent collapsing of lung alveoli in case of patients suffering from asthma, and also deep-sea divers (Budria, Bara et al. 1989). These surfactants reduce alveolar surface tension, and this allows reduction in the work needed for breathing and helps stabilize the alveoli. Multi-block poloxamer surfactants can be used as additives to suppress protein aggregation and facilitate refolding of denatured proteins in solution (Mustafi, Smith et al. 2008). Bioderived surfactants are widely used to synthesize drug-bearing nanoparticles and biodegradable scaffolds (Palocci, Barbetta et al. 2007; Lapitsky, Zahir et al. 2008).

Critical Micelle Concentration (CMC) is an important characteristic of surfactants. At low concentrations the surfactant molecules are loosely integrated in the solvent with

some being present at the surface, their hydrophobic part preferentially residing at the air-water interface and thus reducing surface tension. At the CMC there is monolayer coverage of the air-water interface, and the surfactant molecules in the bulk of the solution begin to form micelles. For concentrations greater than the CMC, more micelles are formed in the bulk but the constitution of the monolayer at the interface no longer changes. Therefore, the surface tension becomes constant at concentrations higher than than CMC. Knowledge of the CMC is necessary for understanding the behavior of solutions that contain surfactants and to ensure optimal use of surfactants in practical applications. Various techniques including spectrophotometry (Ananthapadmanabhan, Goddard et al. 1985; Dominguez, Fernandez et al. 1997; Tran and Yu 2005; Mohr, Talbiersky et al. 2007), measuring surface tension (Simister, Thomas et al. 1992), capillary electrophoresis (Lin 2004), electrical conductivity measurement (Dominguez, Fernandez et al. 1997), and cyclic voltammetry (Mandal, Nair et al. 1988) have been reported for CMC determination.

We introduce here a unique approach to the determination of CMC using the RSS. The RSS system schematically depicted in Figure 3.1 incorporates a hydrophobic film ring deposited on a stationary substrate. An aqueous sample drop placed within the ring will be confined and symmetrically centered due to hydrophilic-hydrophobic repulsion. The drop will assume a hemi-spherical shape if a ring of the requisite inner diameter is used. Convection is afforded by one or more miniature air jets directed tangentially (Cserey and Gratzl 1997; Shetty, Syed et al. 2005; Kao, Hsu et al. 2008) or in a perpendicular fashion as in this work (Figure 3.1), at the surface. Thus, convection in the drop depends on mechanical air-liquid coupling.

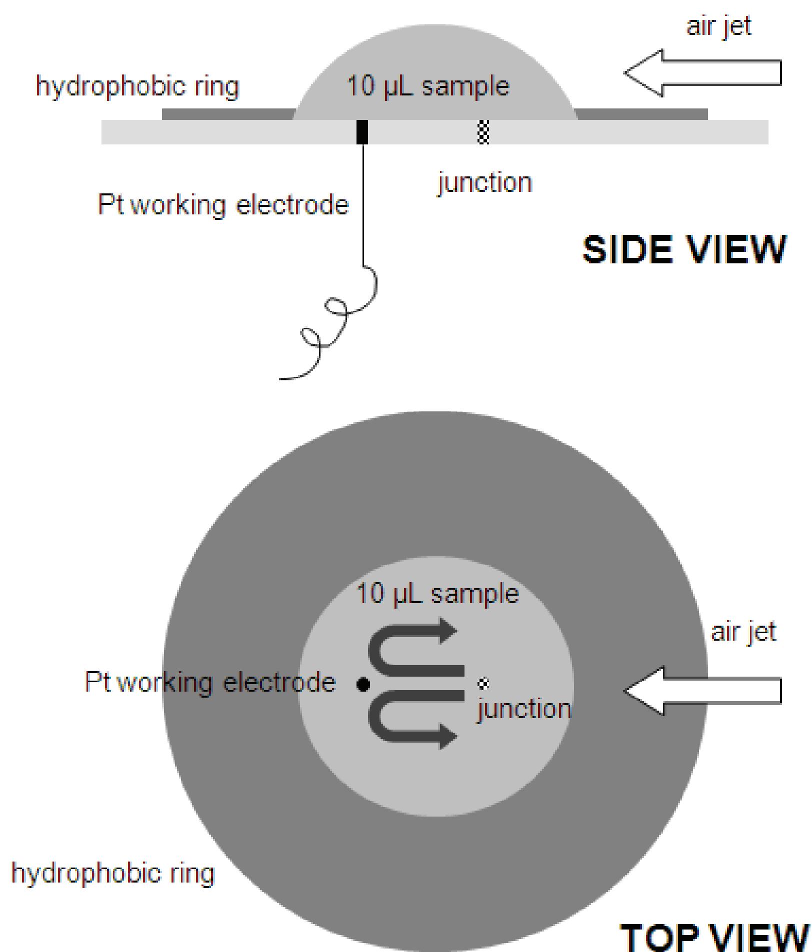


FIGURE 3.1. Schematic view of the Rotating Sample System used for CMC detection. A 10 microliter aqueous sample drop is surrounded by a hydrophobic ring on glass substrate. The substrate contains an embedded Pt working electrode and a junction. The dark arrows in the top view represent flow patterns generated in the drop by a single perpendicular air jet. These patters have been verified visually using microscopic colloid particles obtained from ink, suspended in a droplet. Movement of these particles exhibited similar flow patterns (not shown) as those indicated by the dark arrows.

Vigorous convection in microliter drops is difficult to achieve even via direct contact with some type of a moving mechanical component. Yet efficient hydrodynamic performance in 20 microliter samples has been achieved in multiple studies using the RSS that incorporates no moving mechanical parts. We have demonstrated using diffusion for reagent delivery into microliter drops electrochemical and optical microtitrations that require thorough mixing (Xie and Gratzl 1996; Hui and Gratzl 1997). Trace metal (Hg, Pb) detection with anodic stipping voltammetry (Cserey and Gratzl 1997; Shetty, Syed et al. 2005) indicated that the RSS is equivalent to the rotating disc electrode (RDE) but in very small solution volumes. Recently, we reported on electrochemical micro-pH-stating for determining enzyme activities with the RSS platform (Kao, Hsu et al. 2008).

Owing to the unique way of generating hydrodynamic conditions, i.e., via surface-air mechanical coupling, the RSS can be used to study air-liquid interfaces. This requires, however, that changes in the intensity of convection for the same air-jet velocity can be assessed. Electrochemistry is sensitive to flow because it modulates the thickness of the diffusion layer adherent to the electrode, and to bulk viscosity that influences the rate of diffusion to and from the electrode. Thus, a suitable small electrode embedded in the substrate within the ring, and a junction in the substrate under the drop will enable the electrochemical indication of modulations of flow and viscosity due to the presence of surfactants.

The RSS is in a unique position to probe both the solution bulk (Xie and Gratzl 1996; Cserey and Gratzl 1997; Hui and Gratzl 1997; Shetty, Syed et al. 2005; Kao, Hsu et al. 2008) and the sample surface both of which provide useful information about

different specimens. In this work, the RSS platform incorporating a single air jet perpendicular to the surface of the sample drop (Figure 3.1) is used for examination of rheological properties of aqueous solutions containing Triton X-100, a common surfactant molecule, and for the determination of the CMC of this molecule with both stationary and hydrodynamic voltammetry.

3.3. Experimental

3.3.1. Materials

All chemicals were from Sigma (St Louis, MO); solutions were made with 18 MΩ Milli-Q water (Milli-QUV plus from Millipore, Billerica, MA). The samples were characterized electrochemically using freshly prepared analytical grade potassium ferrocyanide. A stock solution of Triton X-100 (2000 ppm) in KNO_3 (0.1 M) was used to prepare different dilutions. Each sample contained a final composition of ferrocyanide (25 mM), KNO_3 (0.1 M), and Triton X-100 (varied from 0.1 to 800 ppm).

3.3.2. Apparatus

Electrochemical measurements were made using a CHI660 electrochemical workstation (CH Instruments, Austin, TX). The Rotating Sample System, RSS, was fabricated as described in previous work (Shetty, Syed et al. 2005). The electrochemical micro-cell (Figure 3.1) consisted of a platinum mini-disc working electrode (WE) made from 250 μm diameter platinum wire (Alfa Aesar, Ward Hill, MA). The WE disc is

embedded flush in the glass substrate together with a junction filled with agar gel (1 wt% Type I, Sigma). Silicone elastomer (DOW Corning, Midland, MI) was applied to form the hydrophobic ring that confines the sample drop into a near-semi-sphere. Electrodes were polished with alumina paste (Buehler; 1- and 3-micron) on Microcloth polishing pad (Buehler, Lake Bluff, IL) mounted on a Delta 31-120 disk sander (Delta, Jackson, TN). A Ag|AgCl (3M KCl) reference electrode (BAS, West Lafayette, IN) and a gold wire counter electrode (Alfa Aesar) were placed under the substrate in KNO_3 (0.1 M), connected to the sample by the junction. Capillary tubes (wall thickness 0.025 mm, i.d. 0.5 mm; A.H. Thomas Co., Philadelphia, PA) were used to create an air jet to induce flow in the sample. One air jet was used in this work, directed perpendicular to the surface and parallel to the substrate as shown in Figure 3.1. The flow rate of the air jet was 160 mL/min in all measurements at the nozzle placed 4 mm from the base of the sample drop.

2.3.3. Procedures

Cyclic voltammetry, CV, experiments were performed in both stationary and rotated samples containing Triton X-100 over a wide range of concentrations (0.1 to 800 ppm). In order to minimize sample evaporation, the air used for rotating the sample was driven through porous air stone in deionized water to produce fine air bubbles with a large total surface area for humidification. Two controls containing no surfactant have been measured prior and after each measurement. The average of these two controls was used to normalize the actual measurements.

3.4. Results and Discussion

As the RSS affords convection to the liquid micro-sample through mechanical coupling of its surface with air flow, changes in surface tension as well as bulk viscosity are reflected in the cyclic voltammograms (CVs) of rotated samples. To probe the effects of the presence of Triton X-100 on voltammetry electro-oxidation of ferrocyanide was used to avoid potential interference by oxygen that could occur if reduction of ferricyanide were to be used for the measurements. It is noted that no interference by the cyanide complex with micelle formation was found in earlier work (Mandal, Nair et al. 1988).

The sample size was reduced in this work from the typical 20 microliter (Xie and Gratzl 1996; Cserey and Gratzl 1997; Hui and Gratzl 1997; Shetty, Syed et al. 2005; Kao, Hsu et al. 2008) to 10 microliter in order to minimize spreading of the drop beyond the inner edge of the hydrophobic ring at higher concentrations of Triton X-100. The 10-microliter samples deposited onto the RSS, however, had a tendency to some degree of variation in shape as the function of the concentration of the surfactant. When two antiparallel tangential air jets were used for rotation as in previous work (Shetty, Syed et al. 2005; Kao, Hsu et al. 2008) these small variations in shape introduced noticeable inconsistency in the measurements. This was the rationale for adopting the perpendicular single-air-jet arrangement shown in Figure 3.1 in this work. The flow patterns generated this way are different from rotational flow about a single vertical axis as in the usual RSS setup (see dark arrows in the drop in Figure 3.1, top view) yet they generate sufficient convection in the drop to ensure that the measured CVs are sygmoidal in shape as expected in hydrodynamic voltammetry (Bard A.J. and Faulkner 2001). This

modification has minimized variations in flow velocity that were previously caused by droplet deformation and thus, better reproducibility in the recorded data could be achieved (overall standard deviation was 2.7% including all experiments).

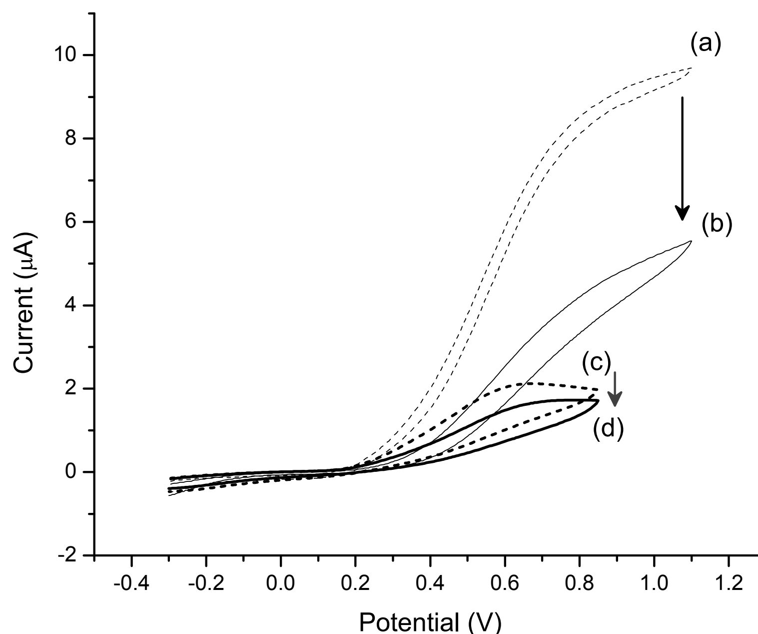


FIGURE 3.2. Cyclic voltammograms of 25 mM $K_4[FeCN_6]$ at the CMC of Triton X-100 (140 ppm). The CVs of the Triton X-100 (140 ppm, CMC), in both rotated (thick lines) and non-rotated (thick lines) sample drops with their controls (dashed lines). A 250 μm Pt disc electrode was used as working electrode (WE). The plateau current in a rotated sample at the CMC (b) is about 57% of its control (a); the peak current in the non-rotated sample of the same concentration (d) is about 77% of its control (c). The peak currents are self-referenced to the respective baselines measured at 0.15 V.

Figure 3.2 shows CVs obtained at 140 ppm surfactant level as typical examples for rotated and stationary samples, respectively. In general, CVs from stationary samples show the usual oxidation and reduction peaks. The peak current was determined as the difference between current at 0.6-0.7 V minus current at 0.1-0.2, to self-reference these data. The data derived from CVs measured in rotated drops are plateau currents, measured at 1.1 V. The acquired current values were subsequently normalized relative to their respective controls, i.e., voltammograms measured in solutions containing no surfactant. The resulting normalized currents over the entire studied range of concentrations of Triton X-100 (0.1 - 800 ppm) are summarized in Figure 3.3. (We note that the surfactant in the absence of potassium ferrocyanide did not show electrochemical activity, corroborating a similar observation by Mandal *et a*l., 1988.)

The absolute currents in the rotated drops are typically about four times higher than currents obtained in stationary samples. Plotting normalized currents is, however, obviously more useful because parallel as well as divergent tendencies between flow and no-flow in the drop can be more clearly seen.

To interpret the results shown in Figure 3.3 it is necessary to consider the differences between stationary and hydrodynamic voltammetry and the ways they are influenced by viscosity, bulk flow, and adsorption onto the electrode. In stationary samples, ferrocyanide is transported to the surface of the working electrode via diffusion. The diffusion coefficient, D, is modulated by viscosity, η, according to the Einstein-Smoluchowski equation (Islam 2004):

$$D = \frac{kT}{6\pi a\eta} \tag{3.1}$$

where k is the Boltzmann constant, T is the absolute temperature, and a is proportional to the size of the molecule and the adherent hydrate shell. Increasing viscosity therefore results in decreased current. The other factor that may contribute to a current decrease is adsorption of the surfactant onto the electrode surface that would reduce the effective electrode area. This factor, however, is unlikely to play a role in modulating the observed currents. This is because current in stationary samples begins to increase again after the CMC. It would be counterintuitive to assume that the electrode surface is less covered by adsorbed molecules at much higher surfactant concentrations. Therefore adsorption onto the electrode surface is not considered in this discussion.

Viscosity has the same effect on the diffusion rate in both stationary and rotated samples. It is therefore reasonable to assume that this effect does not differ between rotated and non-rotated drops. Currents measured in hydrodynamic conditions, however, reflect viscosity also through its effect on flow rate. This will result in additional decrease in current for the same increase in viscosity. In the RSS there is an additional effect of surfactant molecules that can modulate electrochemistry: via air-water coupling at the drop's surface.

Cyclic voltammograms in rotated samples containing 25 mM potassium ferrocyanide and different concentrations of the surfactant Triton X-100 showed decreasing plateau currents with increasing surfactant concentration. It is well known that surfactants reduce the surface tension of the air-water interface. However, beyond a

certain concentration known as the Critical Micelle Concentration (CMC), there is no further decrease in surface tension. In Figure 3.3, point D (140 ppm or 0.24 mM) where the plateau current does not decrease further indicates the CMC of Triton X-100, being in good agreement with reported values obtained with other methodologies (Courtney, Simpson et al. 1986; Mandal, Nair et al. 1988; Mohr, Talbiersky et al. 2007). This finding could be interpreted solely by considering that flow is modulated by air-sample mechanical coupling that varies according to surface tension, and thus should no longer decrease beyond the CMC. The experimental results that we obtained with the RSS, however, cover a broader concentration range than what has been studied before (40 - 400 ppm) (Mandal, Nair et al. 1988) and reveal a number of further features in addition to the CMC. Comparison of plateau currents in rotated samples with the respective peak currents in stationary drops adds further detail to the analysis, as described below.

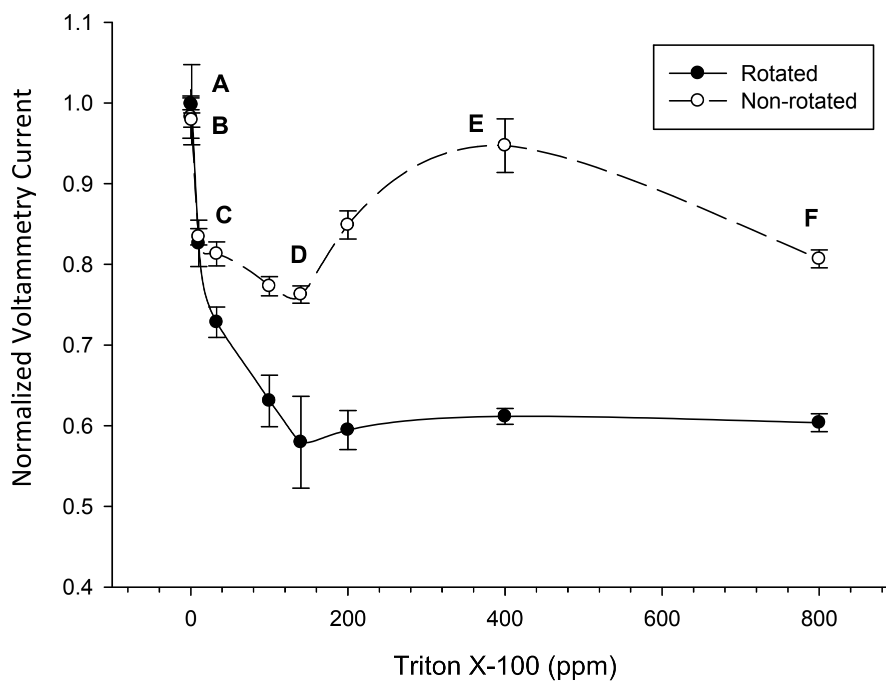
The curves of normalized currents plotted *versus* a linear concentration axis (Figure 3.3a) show an abrupt decrease in both rotated and stationary samples from no surfactant content to 10 ppm of Triton X-100. This range is shown “enlarged” in the semi-logarithmic plot. From the figure it is clear that, interestingly, there is no appreciable decrease in current up to 1 ppm, i.e., from point A to B. At B currents in both rotated and stationary samples begin to decrease. The overall tendencies and even the actual values of the normalized currents are very similar in both the hydrodynamic and stationary drops up to 10 ppm (C). This should imply that up to point C only the effect of viscosity on diffusion plays a role but flow is not influenced by the increasing surfactant concentration.

At surfactant concentrations above point C (10 ppm) the two plots part: the plateau current continues to decrease in the rotated drop at the same rate (on a logarithmic scale) but current in a stationary drop decreases at a lesser rate. Since the latter is indicative only of viscosity, we can infer that viscosity increases at a lesser rate beyond C. The additional decay in rotated samples must therefore be due to reduced flow. This is probably caused by both higher bulk viscosity and weaker air-water coupling due to decreasing surface tension.

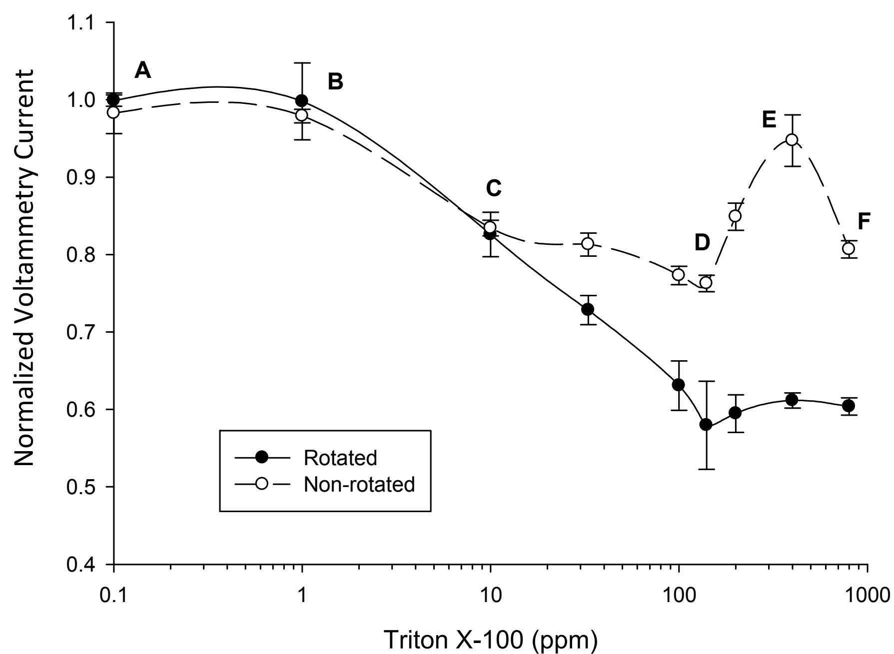
The normalized current in rotated samples continues the same trend up to the sharp minimum observed at point D (140 ppm or 0.24 mM). This is identified as the CMC of Triton X-100 where the surface is saturated and thus, air-sample mechanical coupling becomes constant. However, this coincides with a minimum observed in stationary samples which indicates a concurrent change in viscosity. This is attributed to the formation of micelles.

Above the CMC the peak current in stationary drops sharply increases up to point E (400 ppm), in parallel with a much smaller increase in a rotated drop. Taken together these observations indicate that viscosity decreases after the CMC but air-water coupling remains the same. This latter is to be expected as beyond the CMC surface coverage is complete and thus it no longer changes. Decreasing viscosity with increasing micelle concentration may be explained by considering that ferrocyanide partitions into micelles where viscosity in the surfactant phase is likely lower than in water. Thus an increasing fraction of the trajectories of thermal motion of the probe molecules lie within the surfactant phase. This would result in an overall decrease in apparent viscosity in the composite solution.

The decay in peak current above point E implies again an increase in viscosity. The plateau current in rotated samples, however, remains essentially constant. The large number of micelles present in this concentration range may change rheology of the colloid slution and thus flow, such that the effect of decreasing diffusion coefficient may hypothetically be compensated for by a decrease in the diffusion layer thickness. To arrive at a definite interpretation of the behavior of relatively concentrated solutions of Triton X-100 will, however, require further studies.



(a) linear scale



(b) log scale

FIGURE 3.3. Normalized cyclic voltammetry currents of 25 mM $K_4[FeCN_6]$. The obtained CV currents are normalized relative to their respective controls measured at a 250 μm Pt disc electrode in both non-rotated and rotated 10 microliter samples. The CMC value of Triton X-100 corresponds to the minimum in both rotated and non-rotated samples at 140 ppm (0.24 mM). The currents shown are normalized peak currents for stationary drops and plateau currents for rotated drops as explained in Figure 3.2.

3.5. Conclusions

In this work we have demonstrated the utility of the Rotating Sample System as a simple tool to determine the Critical Micelle Concentration of the surfactant Triton X-100. In addition, insight into the structure of the air-water interface as well as the bulk of the solution could be inferred over a wide range of concentrations from the results. This was aided by performing identical measurements in rotated as well as stationary samples. The approach introduced in this work is thus well suited to study nonionic surfactants.

Overall, the RSS enables surface and rheological studies conducted in very small samples. This is because of the unique way it generates hydrodynamic conditions: via air-water mechanical coupling. Modulations in this coupling by surfactants can be indicated with electrochemical measurements using an electro-active probe molecule that does not significantly affect surface tension or bulk rheology. Preliminary studies conducted in our laboratory indicate that in addition to surfactants solutions containing proteins and/or lipids can also be studied with the RSS. That extremely small samples suffice is advantageous when sample is costly or obtaining larger specimens is problematic such as in biomedical experiments involving small laboratory animals, and in other areas where the amount of samples is limited.

3.6. Acknowledgement

This work was supported by NSF 0352443.

3.7. References

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Chapter 4

Serum Cholinesterase Assay Using a Reagent-free Micro pH-stat

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This manuscript demonstrates that this system is suitable for use in biological specimens. We tested fetal bovine serum as a model specimen and also utilized human serum to mimic clinical tests. The results obtained in the manuscript suggest that the reagent-free micro pH-stat system we have developed can be employed in point-of-care diagnostics.

4.1. Abstract

Enzyme activities in body fluids are often utilized as diagnostic markers for physiological conditions and diseases. Common enzyme assays use optical methods that often require the use of pseudo-substrates and associated dyes. We introduce here a reagent-free micro pH-stat that can determine absolute enzyme activity without the need for exogenic reagents. This approach employs electrolysis for precise dosing of the requisite acid or base titrant to stat the pH of the sample. The micro pH-stat is based on the Rotating Sample System (RSS), a convection platform for microliter drops. Activities of serum cholinesterase in fetal bovine serum and human serum were analyzed with this approach. The performance of this system is comparable to standard techniques (r^2 = 0.99) yet it offers a broader range of detection. The reagent-free micro pH-stat has potential to be developed as a miniaturized device for point-of-care testing.

4.2. Introduction

Enzyme activities in body fluids such as blood and urine are often utilized as diagnostic markers for physiological conditions and diseases (Panteghini, Bais et al. 2006). In order to determine enzyme activity in a sample the rate of conversion of the substrate to the product of the enzyme reaction needs to be assessed. Optical measurements using spectrophotometry and fluorimetry are the most common methods for enzyme assays. Other techniques such as radiometric, titrimetric, chromatographic, calorimetric and chemiluminescence measurements have also been reported for particular enzyme assays (Eisenthal and Danson 2002). Optical approaches depend on calibration with standard solutions and the use of pseudo-substrates and/or the addition of dyes and other reagents that may interfere with the reaction or constituents of the specimen. An alternative approach is based on pH-stating: a technique that allows for the monitoring of the rate of reactions which induce a pH change in the solution. In a pH-stat constant pH of the sample is maintained at a set level by the continuous addition of the required H^+ or OH^- which counterbalances the pH shift from the ongoing enzyme reaction. The reaction rate therefore can be readily obtained since it is equal to the rate of titrant addition at steady state. Earlier the use of water electrolysis as an alternative to titration with an actual acid or base reagent has been suggested for the electrolytic addition of H^+ or OH^- for enzyme assays in ~100 mL volumes of buffer (Karcher and Pardue 1971; Adams, Betso et al. 1976).

We report here on a novel approach for enzyme assays in microliter samples using electrochemical pH-stating. This approach takes advantage of the Rotating Sample System (RSS) platform that has been introduced in our laboratory as a convective

platform for optical and electrochemical analyses in small sample drops (Diefes, Hui et al. 1996; Xie and Gratzl 1996; Cserey and Gratzl 1997; Hui and Gratzl 1997; Shetty, Syed et al. 2005). The electrochemical micro pH-stat that we propose here incorporates the main concepts of this platform as shown schematically in Figure 4.1. A hydrophobic ring with proper inner diameter holds the 20 μL sample in place confining it into a hemispherical shape by virtue of high surface tension at the air-water interface. Vigorous rotation and mixing of the drop is generated by two anti-parallel air jets tangential to the drop surface. Acid or base addition in our system is achieved by water electrolysis at a mini-disc Pt working electrode which is embedded in the stationary substrate. The magnitude of current is controlled by the actual difference between the real-time pH reading in the sample and the desired pH value set in the control system. We have recently shown that in such a system, with precise current injection and efficient mixing it is possible to maintain the desired pH within +/- 0.05 of the preset pH value in drops of artificial samples of red cell cholinesterase enzyme (AChE, EC 3.1.1.7) prepared in physiological buffer (Kao, Hsu et al. 2008).

The objective of this work was to bring the reagent-free micro pH-stat system a step closer to real-world applications by validating the approach for biological specimens, particularly serum. We analyzed serum cholinesterase (ChE, EC 3.1.1.8) here that often serves as a sensitive indicator of the synthetic capacity of the liver. A 30-50% drop in ChE activity is observed in acute and chronic hepatitis. A decrease of 50-70% occurs in advanced cirrhosis and carcinoma of the liver (Burtis, Ashwood et al. 2006). Furthermore, organic phosphorous compounds that are constituents of many insecticides inhibit activity of ChE. Hence the decay in ChE activity in serum can be used as an early

diagnostic indication of possible insecticide poisoning (Wilson, Henderson et al. 2002; Burtis, Ashwood et al. 2006). A variety of alternative ChE substrates for optical assays has been reported (Kramer and Gamson 1958; Ellman, Courtney et al. 1961; Szasz 1968; Mosca, Bonora et al. 2003). Although optical assays are fairly automated, the lack of standards makes it difficult to compare results between laboratories (Wilson, Henderson et al. 2002). Also, the use of artificial substrate and additional color-changing reagents may interfere with the reaction of interest. For instance Ellman's reagent, DTNB (5,5'-dithio-bis-2-nitrobenzoate), can react with sulfhydryl groups (Ellman, Courtney et al. 1961).

The activity of ChE can be directly measured by pH-stating since acetic acid is produced as a side product. We propose here an electrochemical pH-stating technique that requires only the actual substrate to initiate the reaction and therefore it is independent of other exogenic reagents. Given that OH^- is continuously delivered by electrical current and nearly 100% current efficiency is achieved in serum, the method provides an absolute measurement and thus calibration with standard solutions is not necessary. Our results indicate that the accuracy and precision of the electrochemical pH-stat assay is comparable to standard techniques ($r^2 = 0.99$). Minimal sample consumption and that the requisite instrument can be miniaturized render the micro electrochemical pH-stat a simple, cost effective, and potentially portable system for enzyme assays.

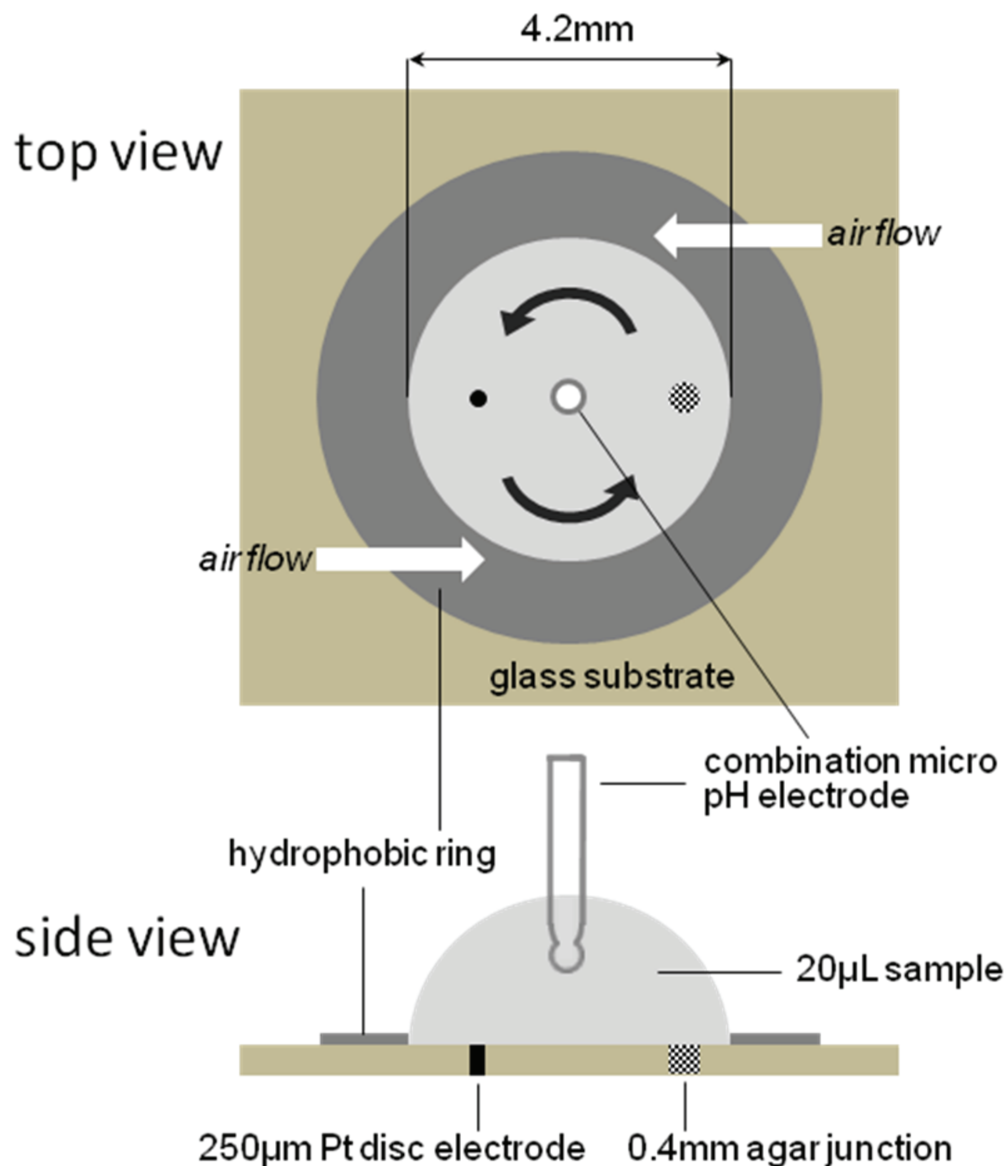

FIGURE 4.1. Schematic of the electrochemical micro pH-stat built on the Rotating Sample System platform. A 20 µL sample drop is surrounded and confined by a hydrophobic ring on a glass substrate containing an embedded Pt disc electrode and a junction. The dark arrows represent flow patterns generated in the drop by two tangential anti-parallel air jets. A combination micro pH electrode is immersed to a depth of 1 mm from the top into the sample. The schematics are approximately drawn to scale.

We note that electrochemistry in specimens of biomedical origin is generally challenging due to the complex composition of the sample that can lead to contamination of the electrode surface and/or competing oxidation or reduction of constituent molecules especially at the high overpotentials needed for water electrolysis. In addition, changes in viscosity and surface tension in serum can influence the hydrodynamics of the RSS and the strong buffer in serum may also reduce the sensitivity of pH-stating. Despite these technical challenges, the quality of the data obtained in this work is still similar to the one in artificial buffer seen in our earlier publication (Kao, Hsu et al. 2008). Therefore it is an important argument for using galvanostatic methods in analyses of biological fluids.

4.3. Experimental

4.3.1. Materials

All chemicals were from Sigma (Sigma Chemical Co., St. Louis, MO, USA) and Fisher (Fisher Scientific, Pittsburgh, PA, USA). All aqueous solutions and subsequent dilutions were prepared with 18.2 MΩ-cm deionized water (Millipore Milli-QUV plus, Billerica, MA, USA). Substrate solutions were prepared fresh before the experiments. Serum samples stored in a refrigerator are stable for 7 days. Native and heat inactivated fetal bovine serum (FBS) were from HyClone Laboratories, Inc. (Logan, UT, USA).

4.3.2. Apparatus

The fabrication of the micro pH-stat cell (Figure 4.1) has been described in previous work (Kao, Hsu et al. 2008). A 250 μm diameter Pt mini-disc electrode (Alfa Aesar, Ward Hill, MA, USA) was employed as working electrode. 1% w/w Type I agarose was prepared in heated 0.1 M KNO_3 solution and filled into the junction hole embedded in the glass substrate. A hydrophobic ring of 4.2 mm inner diameter for sample positioning and confining it to a hemispherical shape (Figure 4.1) is made of painted silicon elastomer (DOW Corning, Midland, MI, USA). A micro pH-electrode (MI-4154 Microelectrodes, Inc., Bedford, NH, USA) with a 1 mm diameter tip was immersed at the vertical axis of the sampled drop to a depth of 1 mm for real-time pH monitoring.

4.3.3. Procedures

Determination of current efficiency in the electrochemical micro pH-stat: We measured current efficiency by adding 5 μL of HNO_3 (2-8 mM) to lower the pH of 15 μL FBS. A constant current was subsequently applied to neutralize the acid and bring the pH of the sample back to its original value. Once the original pH was restored, the duration of current injection was recorded and then current efficiency was obtained by using Faraday's law. We define current efficiency as the percentage of moles of acid added to the sample divided by the added charge necessary to restore the initial pH.

ChE assay in fetal bovine serum with electrochemical pH-stating: 0.9-2.4 U/mL cholinesterase (EC 3.1.1.8) from equine serum was prepared in heat inactivated FBS.

0.15% Intralipid (Baxter Healthcare Corp., Deerfield, IL, USA) was added to the enzyme solution to ensure effective mixing (Shetty 2005). The substrate acetylthiocholine iodide (ATCh) was prepared in 0.1 M NaCl and adjusted to pH 8.

The ChE assays were performed at room temperature. 15 μL substrate solution was placed on top of the RSS cell. Once the pH reading was stabilized at 8, 5 μL of 10-100 folds diluted ChE solution was added to the substrate solution. The protons generated by the enzyme reaction were neutralized by OH^- titration, generated electrolytically by the micro pH-stat system. In each pH-stating experiment the sample pH was maintained within 0.1 pH unit of the preset pH for one minute. The enzyme activity was obtained from averaging the current over 1 min of steady state pH-stating and applying Faraday's law to convert current into molar amount.

Spectrophotometric control assay: The ChE assay using Ellman's procedure has been adapted as control (Ellman, Courtney et al. 1961). All the solutions except the enzyme solution were prepared with 0.1 M phosphate buffered saline (PBS) with the pH adjusted to 8. ChE solutions from 0.9-2.4 U/mL were prepared in FBS. A 0.5 mL mixture of 0.1 mL DTNB (final concentration 0.3 mM), 0.1 mL ATCh (final concentration 1.5 mM), and 0.3 mL diluted ChE in FBS (100-fold) was analyzed using a spectrophotometer (Gilford Response, Oberlin, OH, USA) at room temperature. Readings were taken at 412 nm. The extinction coefficient used for DTNB is 13600 $M^{-1}cm^{-1}$.

It should be noted that we observed strong residual cholinesterase activity in native FBS and therefore heat inactivation to eliminate residual enzyme is needed prior to the enzyme addition.

ChE assay using human serum with both methods: Blood sample of a volunteer was drawn and serum samples prepared for analyses at the clinical laboratory of the University Suburban Health Center (Cleveland, OH, USA). ChE activity in these samples was analyzed with the pH-stat assay using the protocols described above. For comparison, the serum was also sent to LabCorp (Burlington, NC) for ChE testing.

4.4. Results and Discussion

4.4.1. Current efficiency of the electrochemical micro pH-stat in fetal bovine serum

The current efficiency of electrolysis quantifies the actual amount of OH^- generated per charge. Current efficiency in the reagent-free micro pH-stat in serum was found to be 100.4±0.5% (with at least 6 parallels) indicating that there is no current loss in OH^- generation. This result is consistent with the fact that oxygen is the only electro-reducible molecule that is present at significant concentrations in serum (besides water). The electro-reduction of O_2, however, yields the same amount of OH^- per electron as water electrolysis (Kao, Hsu et al. 2008). Therefore no current loss occurs even in the presence of O_2. This is important as it enables calibration-free, absolute measurements with this platform.

The term "calibration-free" in this context is used in comparison to optical assays. Due to essentially 100% current efficiency of OH^- generation in serum, enzyme activity determination requires only the measurement of steady state current that directly translates to the information sought. Thus, no prior calibration performed with standard

solutions is needed as opposed to in optical methodologies. Also, optical interferences may vary from specimen to specimen that is hard to account for by calibration. The calibration measurements and the problem of interferences are avoided in our approach by just having to measure a current value. As it is safe to rely on modern electronics to measure absolute currents accurately and precisely, the methodology presented in this work can be said to be absolute in comparison with the optical approach.

4.4.2. Effect of sample dilution on current efficiency

Diluting enzyme samples is a common procedure in enzyme assays. There are three main reasons for the dilution: (1) sufficient dilution reduces the turbidity of the samples so that optical absorbance-based methods can be used for the measurements; (2) proper dilution is necessary to ensure that the optical signal falls within the linear range of detection throughout the measurement; (3) enzyme dilution slows down the rate of consumption of substrate thus providing an extended time window for observation of steady-state enzyme kinetics. Only this last reason for dilution needs to be considered if using the pH-stat assays reported here because of its independence of sample turbidity and its broad detection range (3 orders of magnitude in the system reported here). Therefore, the range of sample dilution can be very flexible.

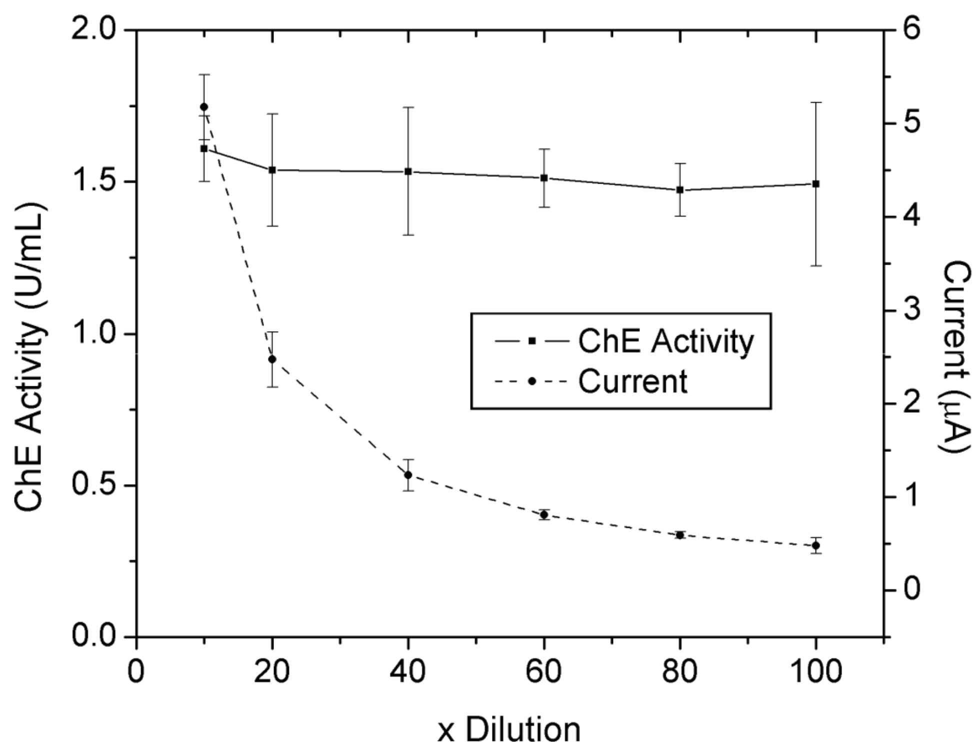

FIGURE 4.2. Effect of enzyme dilution on the enzyme activity as determined with the micro pH-stat. A stock solution of ChE in FBS was diluted with 0.1 M NaCl from 10 up to 100 fold. ChE activity was measured by electrochemical pH-stating at pH 8, room temperature. The measured currents are indicated with dashed line with the corresponding scale on the right axis. The back-calculated enzyme activities are indicated with solid line with the corresponding scale on the left. Results are averaged over at least three parallels.

ChE activities (~1.5 U/mL at room temperature) in serum samples across a series of dilutions (from 10 to 100 fold) were measured with the micro pH-stat (Figure 4.2). The dashed line indicates the actual current required to generate OH^- for pH-stating. The back-calculated ChE activities (indicated with solid line) are obtained by multiplying the currents needed for pH-stating with the respective dilutions. The fact that the activities of ChE have been consistent even at greater dilutions indicates that no appreciable loss of enzyme activity due to adsorption at the liquid/air interface and onto the electrodes occurs. These results also demonstrate that the accuracy and precision of this system are consistent in a broad range of dilutions which implies that (1) sample mixing is efficient; (2) the control system accurately adapts the current output according to real-time pH feedback; (3) current density is high enough to ensure nearly theoretical current efficiency even at 100 fold dilution.

4.4.3. Determination of ChE enzyme activities in fetal bovine serum with electrochemical pH-stating

Electrochemical analysis in biological fluids such as in serum is generally difficult due the presence of various substances especially proteins and lipids (Guidelli, Aloisi et al. 2001; Zhang, Chi et al. 2002). Contamination from lipids and non-specific adsorption of proteins can decrease the active surface area of electrodes. This results in loss in sensitivity in potentiostatic measurements. In this work, however, the electrochemical pH-stat functions as a galvanostat. Therefore, the reduction in electrode surface area may accordingly increase the interfacial impedance but the amount of

required current can still be injected if an adequate driving force is provided. The current source designed in this work is capable of withstanding sample impedances up to 100 kΩ which is sufficient to overcome a minor increase in impedance due to eventual surface contamination.

We tested the electrochemical micro pH-stat system in FBS for measuring the activity of ChE. Figure 4.3 represents the correlation found between ChE activities obtained with electrochemical pH-stating and with the standard spectrophotometric technique. The data show an overall good agreement between the two methods (r^2=0.99) though at some points, a somewhat larger spread in the results of pH-stat assay is observed. The possible source of these errors may be the natural buffer capacity of serum. Although the pH during the assay was controlled to remain within 0.1 pH unit of the set pH, the presence of strong native buffering in serum may contribute to some variations in the amount of injected OH^- if there is a residual pH difference between the starting and end pH. The buffer capacity of FBS used in this work was 7.46×10^{-4} milliequivalent base to an acid shift of 0.1 pH unit in 1 mL FBS as determined by the electrochemical pH-stat device. A 0.03 pH drift between the starting point and the end point during a one minute pH-stating would then result in 0.22 additional enzyme activity. This would cause a ~10% error in some cases.

We note that any error stemming from the higher buffer capacity of serum can be minimized as follows: (1) Use longer assay time. If the assay is extended to 2 minutes, for instance, the same error will be averaged and reduced by half. (2) Minimize mismatch in pH between the starting point and end point of the pH-stat assay. As buffer capacity is reversible, this error will only occur if there is a discrepancy between the starting pH and

the final pH. Any fluctuation in pH during the measurement will not contribute to the error discussed here.

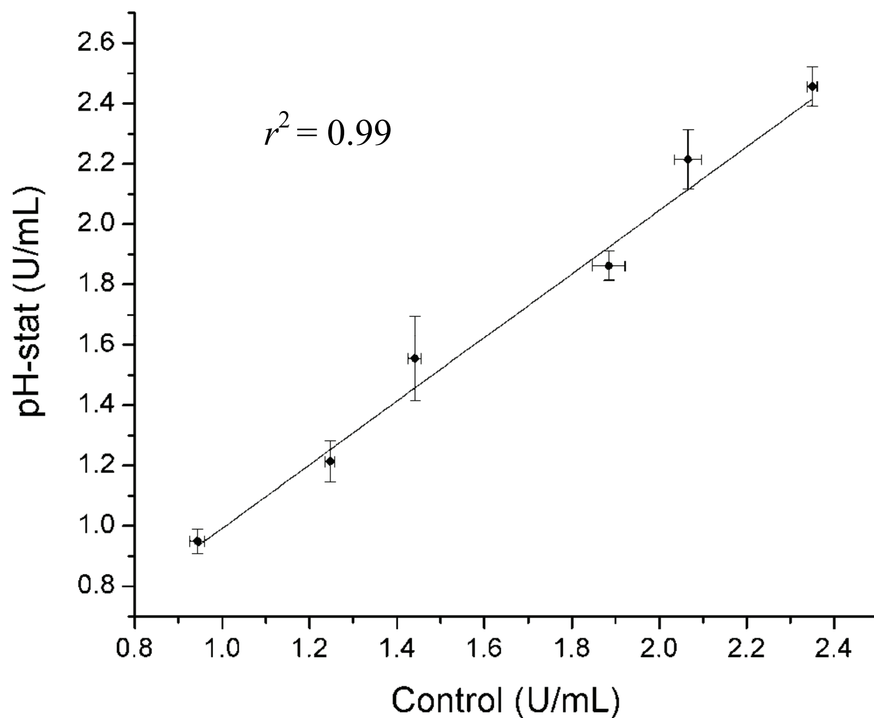

FIGURE 4.3. Comparison of ChE activities in FBS measured with the electrochemical micro pH-stat and with the standard optical assay using Ellman's reagent. The dilutions of the ChE samples with 0.1M NaCl for the pH-stat and the optical assay were 20 and 100 fold, respectively. The measurements were performed at pH 8, room temperature. The correlation coefficient between the two methods is 0.99 ($n \geq 3$).

4.4.4. Determination of ChE activity in human serum with electrochemical pH-stating

We further investigated the feasibility of using the electrochemical micro pH-stat assay for human serum samples. The average ChE activity in the assayed human serum specimen was 2.42±0.2 U/mL by using the same pH-stating protocol as described above. The standard test result from LabCorp is 2.32 U/mL, which is reasonably close to the pH-stat results obtained with our system. Both test results fall well within the reference interval of normal ChE levels (1.9-3.8 U/mL).

4.5. Conclusions

This work demonstrates feasibility of electrochemical pH-stating in microliter serum samples for ChE assay. This approach is unique in that the OH^- required for stabilizing the pH at its set level is "injected" by current and thus no reagent is needed for titration. Also, a wide detection range is available due to the ease of manipulating current output. Efficient mixing that is required for the feedback system to achieve proper pH-stating is made possible by adopting the Rotating Sample System (RSS) platform. An important finding in this work is that the various molecules present in serum do not reduce the current efficiency at the cathodic potentials required for OH^- generation. Precaution is needed when samples with strong buffer capacity are analyzed since a small pH drift between the beginning and the end of the assay may lead to noticeable errors.

Although most enzyme reactions shift the pH in the acidic direction, it is noteworthy that in the case of enzyme reactions shifting the pH in basic direction, at least

+1.2 V is required to produce H^+. Besides water, high anodic potentials can oxidize also common substances present in serum such as chloride, ascorbate, and uric acid. Therefore, loss in current efficiency may occur and calibration for H^+ generation may be necessary in those cases.

We used ChE as a demonstration here but this same platform can be employed for other enzyme assays that involve acid pH shift. Given the advantages of small sample size, absolute measurement, and that there is no need for an actual acid or base reagent the electrochemical micro pH-stat system has the potential to be developed as a portable and calibration-free device for point-of-care testing.

4.6. Acknowledgements

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Chapter 5

Conclusions and Future Work

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5.1. Summary

The RSS has been developed and characterized prior to this work. My personal contribution is to realize the concept of a reagent-free pH-stat based on the RSS. The pH-stat system has been constructed, characterized, and optimized for enzymatic analysis in this work. More importantly, I discovered an innovative clinical application, POCT using reagent-free pH-stat that opened up a new direction for future research. In addition to the development of pH-stat, I also completed a project initiated by a former student, Gautam Shetty, which is an application the RSS for the determination of critical micelle concentration (CMC).

Several important milestones have been accomplished to achieve the goal of developing POCT device for rapid enzymatic analysis. The concept of utilizing electrochemical pH-stating for enzyme assay has been validated and the micro pH-stat system has also been fully characterized (See Appendix I for details). Model enzyme, acetylcholinesterase (AChE, EC 3.1.1.7), prepared in physiological buffer has been tested and the obtained results are of good agreement with standard optical assays (Kao, Hsu et al. 2008). To take this platform a step closer to real-world application, we further validated this approach with serum cholinesterase (ChE, EC 3.1.1.8) in fetal bovine serum (FBS) and human serum. Monitoring for ChE activity in the blood is a common way to track exposure to the dangerous level of organophosphates and carbamates of agricultural workers. The encouraging results of ChE analysis in both FBS and human serum suggest that the proposed electrochemical micro pH-stat is suitable for analysis in biological samples with minimal treatments (Kao and Gratzl 2009).

As a different application of RSS, we also explored the feasibility to detect changes in surface property at the air/liquid interface. We demonstrated using RSS as a simple tool to determine the CMC of the surfactant, Triton X-100 (Kao, Shetty et al. 2008). This is made possible because of the unique feature that forced convection is generated from the surface of the sample.

5.1.1. Proof of concept: electrochemical micro pH-stat

Commercially available pH-stat is carried out by continuously monitoring pH value in the sample while volumetrically adding acid or base to maintain the pre-set pH. Volumetric addition results in constant dilution of the sample; hence, the conventional pH-stat can handle only relatively large sample usually in the order of milliliters. It is difficult to be used in biomedical research because not only the restriction of sample size but also that the use of strong acid/base may potentially damage the biological contents. The electrochemical micro pH-stat system we developed, on the other hand, requires no reagents and the sample size can be 20 μL or less.

Our pH-stat system uses current injection to generate the required hydrogen and hydroxyl ions, the actual current efficiency is therefore critical for quantitative measurements. The protocol we developed to measure current efficiency is externally adding acid/base and then restoring the pH back to original value. This approach effectively eliminates the influence of buffer variables (atmospheric carbonate-bicarbonate buffer from the CO_2 and the strong phosphate buffer in the biological samples) as buffer capacity is reversible. Therefore the charges being delivered must be

the exactly same amount as the acid/base being physically added in order to bring the pH back to initial point. We also found that the 20 μL droplet has a very high surface to volume ratio so the pH can be easily disturbed by carbon dioxide in air and other perturbations from the environment. Hence isolation of samples in a closed chamber and removal of CO_2 from the air flow for sample rotation are important (See Appendix I.4 and Appendix I.5 for details).

Assay of acetylcholinesterase (AChE) has been performed and the results are comparable to the control assay, Ellman's method (r^2 = 0.99, and 0.97). This indicates that the high electrical potential would not affect the catalytic ability of enzymes, and even though the RSS has a relatively large surface-to-volume ratio, loss of activity due to the non-specific protein adsorption is negligible.

The manuscript published in *Analytical Chemistry* (Chapter 2) is an important step to proof the concept and it also built the foundation of this research (Kao, Hsu et al. 2008).

5.1.2. Application of electrochemical micro pH-stat in biological samples

Serum contains complex constituents such as sugar, lipid, mineral ions, amino acids, and proteins. The general concern of performing electrochemical analyses in serum is the non-specific protein adsorption that can foul the electrode surface. However, since the micro electrochemical pH-stat is a galvanostatic approach, minor blockage over the electrode would not affect the sensitivity of measurement. Besides, an important finding

is that various substances presenting in the serum do not reduce the current efficiency during OH^- generation. The only molecule that can be reduced in serum is dissolved oxygen. Fortunately, O_2 reduction produces same amount of OH^- as water-splitting therefore the current efficiency would not be affected. Buffering capacity of sera can induce significant error if there is a mismatch in pH between the starting point and the end point of pH-stat assay. We note that this buffering error can be minimized by extending the assay time or selecting the pH-stat window carefully so that the pH discrepancy between the starting and the end points is minimal.

The manuscript published in *Analytical Biochemistry* confirmed that this system is suitable to be used in biological specimens (Chapter 4). The clinical test using human serum mimics the real-world scenario of diagnosis for toxic exposure. The promising results reported in the manuscript brought the proposed reagent-free micro pH-stat system a step closer to the point-of-care diagnostics.

5.1.3. RSS for determination of Critical Micelle Concentration

As another application of the Rotating Sample System (RSS) besides pH-stat, we demonstrated the feasibility of probing the properties in both the solution bulk and the air/liquid interface (Chapter 3). The idea of using RSS to detect the critical micelle concentration (CMC) is first explored by a previous colleague, Gautam Shetty (Shetty 2005). As the convection in the RSS is induced via liquid/air mechanical coupling, cyclic voltammetry (CV) in the rotated sample exhibit the property changes in both viscosity and the surface tension. However, if we apply an additional CV on the stationary sample,

it would provide information on viscosity only. We can therefore further separate the effects that were resulted from the increase of viscosity and the decrease of surface tension

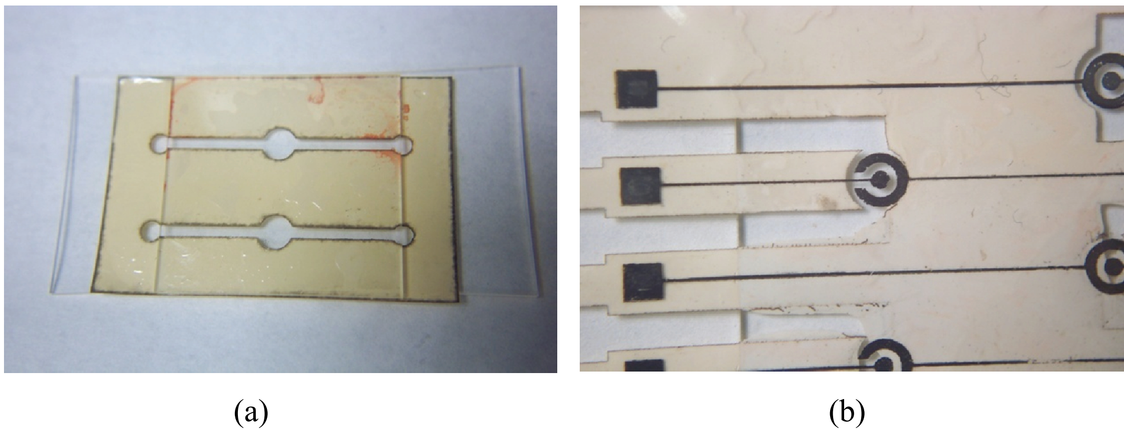
In this work, we also modified the air jet position of the RSS from the edges of the drop to the center of the drop for the first time. This modification is essential to reduce the error resulted from the deformation of the droplet at higher surfactant concentration (Kao, Shetty et al. 2008).

5.2. Future Work

5.2.1. Microfabricated pH-stat chip

Microfabrication technology in general promises many advantages including miniaturization, ease of operation, reliability of instrumentation, and potential for micro-scale, massive parallel analyses. The electrochemical pH-stat cell can be further integrated by thin-film microfabrication technology. Several pH sensitive metal oxides such as those of palladium (PdO_x), platinum (PtO_x), iridium (IrO_x), and ruthenium (RuO_x) have been reported (Liu, Bocchicchio et al. 1980; Kreider, Tarlov et al. 1995; Wipf, Ge et al. 2000; Wang, Yao et al. 2002; Ges, Ivanov et al. 2005; Pocrifka, Goncalves et al. 2006). IrO_x is one of the most promising materials for thin-layer pH electrodes. The advantages of IrO_x in comparison with other pH-senstitive oxides include a wide pH response range (pH 1-13), fast response time (6-15 s), high pH-sensitivity (-59 to 80 mV/pH), long term stability, and most importantly, low sensitivity to interference of biomolecules (Marzouk,

Ufer et al. 1998; Wang, Yao et al. 2002; Ges, Ivanov et al. 2005; Ges, Ivanov et al. 2007). Thin-film IrO_x pH-electrode demonstrated low interference in the presence of Na^+, K^+, Ca^{2+}, Mg^{2+}, dissolved oxygen, lactate, and ascorbate, which renders significant advantage for biomedical applications (Marzouk, Ufer et al. 1998; Marzouk, Buck et al. 2002).



(a) (b)

FIGURE 5.1. Prototype of microfluidic pH-stat chip. (a) A microfluidic chip made by sandwiching two glass substrates with liquid crystal polymer (LCP) as the spacer. The serum sample can be spontaneously drawn into and fill up the entire channel via the openings from either side of the channel. The circular chamber has the internal volume of 1 μL. (b) Thin-film platinum electrodes were deposited on the glass substrate. The electrode surface of the counter electrode (horseshoe-shaped) is much larger than that of the working electrode (dot-electrode) to ensure sufficiently high current density occurring at working electrode.

Figure 5.1 shows the preliminary design of microfluidic pH-stat chip. The volume of the circular chamber in the center of Figure 5.1a is 1 μL. The microfluidic channel is capable of wicking in serum samples and being completely filled up without any air bubble formation. Figure 5.1b represents the micro channel with platinum working electrode and counter electrode deposited on the glass substrate. In the future, thin-film IrO_x pH electrode with planar Ag/AgCl reference electrode (Cheng, Klauke et al. 2006; Sun and Wang 2006) can be integrated into a microfluidic chip for pH-stat assay.

5.2.2. Measurement of substrate concentration and buffer capacity

Provided nearly 100% current efficiency in the sample, the electrochemical pH-stat assay is an absolute measurement and can even be utilized to quantify substrate concentration. Using glucose measurement as an example, the physiological glucose concentration ranges from 50 to 200 mg/dL. One can employ 1 mg/dL glucose oxidase (GOD) from *Aspergillus niger* to catalyze beta-D-glucose to gluconic acid and hydrogen peroxide at pH 5.1, room temperature. The current required for pH-stating will be injected to counterbalance the acid generation due to glucose oxidation. The measurement will be finished once the current required for pH-stating goes to zero, which indicates the glucose in the sample has been totally consumed by GOD. Consequently, the initial glucose concentration within the sample can be back calculated from the overall charges delivered for pH-stating.

In addition to measurement of substrate concentration, the pH-stat technique can also be applied to measure buffer capacity. For instance, in Chapter 4 the buffer capacity

of fetal bovine serum (FBS) has been obtained using the electrochemical micro pH-stat developed in this work.

5.2.3. NADH-based enzyme assay

Nicotinamide adenine dinucleotide (NADH) is an important coenzyme participating in a variety of biochemical reactions. Detection of NADH allows direct monitoring of NADH-linked enzyme reactions. Therefore, amperometric detection of NADH has been under investigation for decades (Jaegfeldt, Torstensson et al. 1981; Gorton 1986). Direct oxidation of NADH at unmodified electrodes requires overpotential as large as 1 V (Jaegfeldt, Torstensson et al. 1981). In addition, unmodified electrode surfaces suffer from electrode fouling due to adsorption of oxidation products on the electrode surface and interference from other electroactive compounds such as ascorbate and uric acid (Raj and Behera 2005).

Many different approaches have been studied in order to reduce this overpotential and to overcome the above mentioned problems by using various mediators and electrode modification methods such as modified graphite electrode (Jaegfeldt, Torstensson et al. 1981), NADH oxidase (NAox) bioassay with hydroxymethyl ferrocene ($FcCH_2OH$) as redox mediator (Serban and El Murr 2006), and self-assembled monolayers (SAMs) of mercaptopyrimidine (MPM) and their derivatives, thiocytosine (TC) and 4,6-diamino-2-mercaptopyrimidine (DMP) on gold electrode for voltammetric detection (Raj and Behera 2005) However, selective electrochemical sensing of NADH is still a difficult task due to the presence of other electroactive species in biological samples. Recently

Prussian Blue (PB) modified electrodes have been reported as a simple method to detect hydrogen peroxide (H_2O_2) at near 0 V versus Ag/AgCl (Moscone, D'Ottavi et al. 2001; Ricci, Amine et al. 2003; Ricci, Amine et al. 2003; Ricci, Goncalves et al. 2003; Ricci and Palleschi 2005). We propose an innovative NADH sensing approach by combining NAox enzyme reaction and low overpotential H_2O_2 sensing on PB modified electrode. NAox provides fast and selective catalytic activity converting NADH to NAD^+ and H_2O_2. NAD^+ regenerated by NAox oxidation offers continuous co-substrate supply for the ongoing redox enzyme reaction. The side product H_2O_2 will be reduced on PB modified electrode at near 0 V applied potential to generate the signal. This method will largely eliminate electrode fouling and interference from other biomolecules since most electroactive biological compounds can only be oxidized but not reduced.

A preliminary experiment can be conducted as the following: Prepare PB modified electrode by placing a 10 μL drop of precursor solution onto the surface of carbon paste electrode. The precursor solution is a mixture of 0.1 M potassium ferricyanide ($K_3Fe(CN)_6$) in 5 μL of 10 mM HCl and 0.1 M ferric chloride in 5μL of 10mM HCl. The electrode is incubated for 10 minutes, then the precursor solution is rinsed away and the electrode is dried in oven at 100 °C for 1.5 hour to obtain a stable layer of PB. Calibration of NADH concentration is performed with 0.1U NAox in PBS solution at -50 mV applied potential versus Ag/AgCl reference electrode. After a stable background current is observed, NADH will be added to the solution and the formation of H_2O_2 will be detected at electrode surface amperometrically. Statistical analysis will be established to evaluate the performance of electrochemical NADH detection.

Once the NADH measurement by using NAox and H_2O_2 detection by PB modified electrode has been accomplished, activities of dehydrogenases which involve generation of NADH can also be measured by this platform. For example, activity of lactate dehydrogenase (LDH) can serve as a model enzyme (Figure 5.2). The amperometric measurement can be operated at constant potential of -50 mV versus Ag/AgCl reference electrode. When a stable baseline is reached, add substrate and record the current due to the formation of NADH. The Michaelis-Menten kinetics of LDH and the results can be compared with the standard reference method from International Federation of Clinical Chemistry (IFCC) (Bais and Philcox 1994).

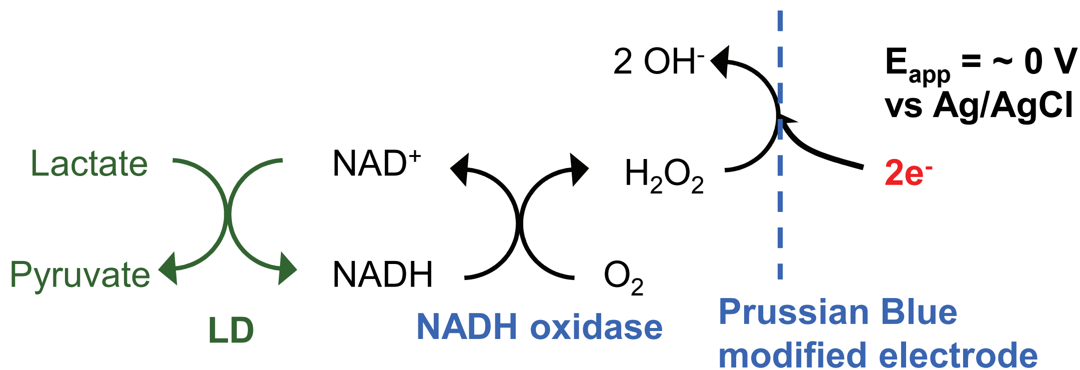


FIGURE 5.2. Schematics of lactate dehydrogenase assay using PB modified electrode. This mediator-based measurement uses coupled enzyme reaction (NAox) to convert NADH to NAD^+ and the released product H_2O_2 can be monitored amperometrically by the PB electrode at very low overpotential (0 to -50 mV).

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Appendix I. Characteristics of the Reagent-Free Micro pH-stat System

A-I.1. Schematics of the Reagent-Free Micro pH-stat

The major components in this system are: (1) LabVIEW program with PID control algorithm; (2) National Instrument DAQ Pad - 6020E; (3) a voltage-to-current (VTC) OPAMP circuit; (4) Fisher AR 15 pH meter with micro pH electrode. Figure A.1 shows a block diagram describing how these components are arranged. An independent Keithley 6430 sub-femtoamp current meter (Keithley Instruments Inc., Cleveland, OH, USA) is connected in series to the sample for monitoring and verifying the actual current passing through the sample.

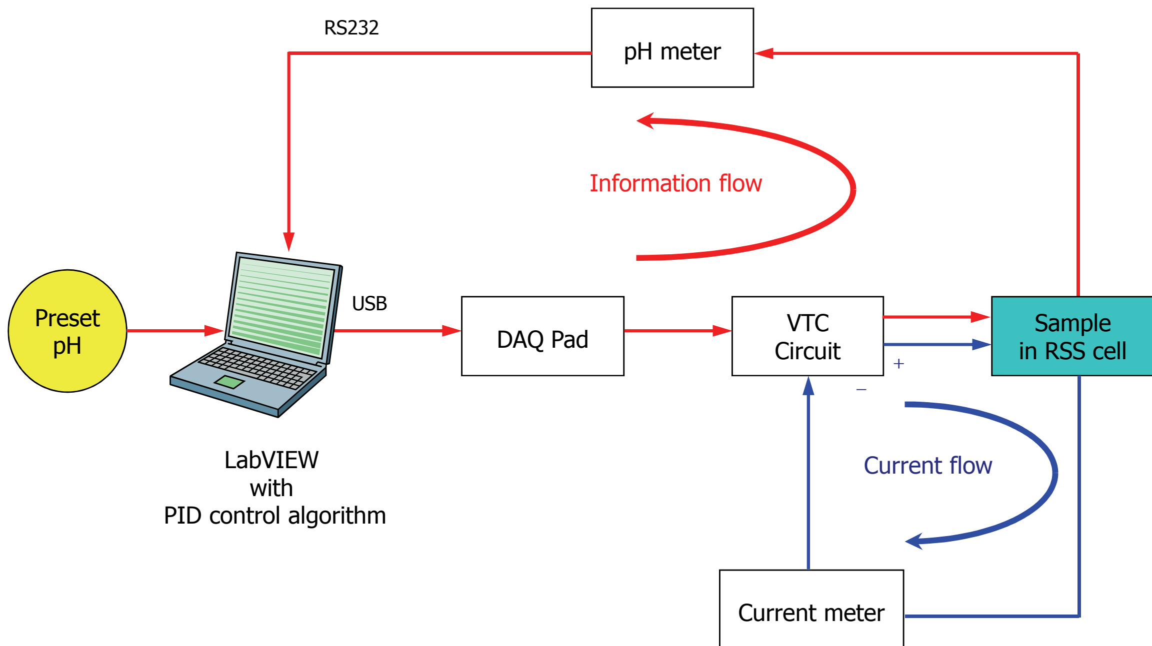

FIGURE A.1. Schematics of the pH-stat system

A-I.2. Current generating circuit and voltage isolation

A voltage controlled variable current source was designed and implemented. The variable current source was constructed using two LM 741 OP AMPS (Figure A.2). The current source was designed such that the current output only depends on the V_{in} and R6 (independent from the sample impedance). It could withstand loads up to 100 kΩ (Typical resistances of samples used in practice is less than 10 kΩ) (Kao, Hsu et al. 2008). This current source was controlled by varying the voltage in the analog channel of a data acquisition card (NI DAQ Pad 6020E).

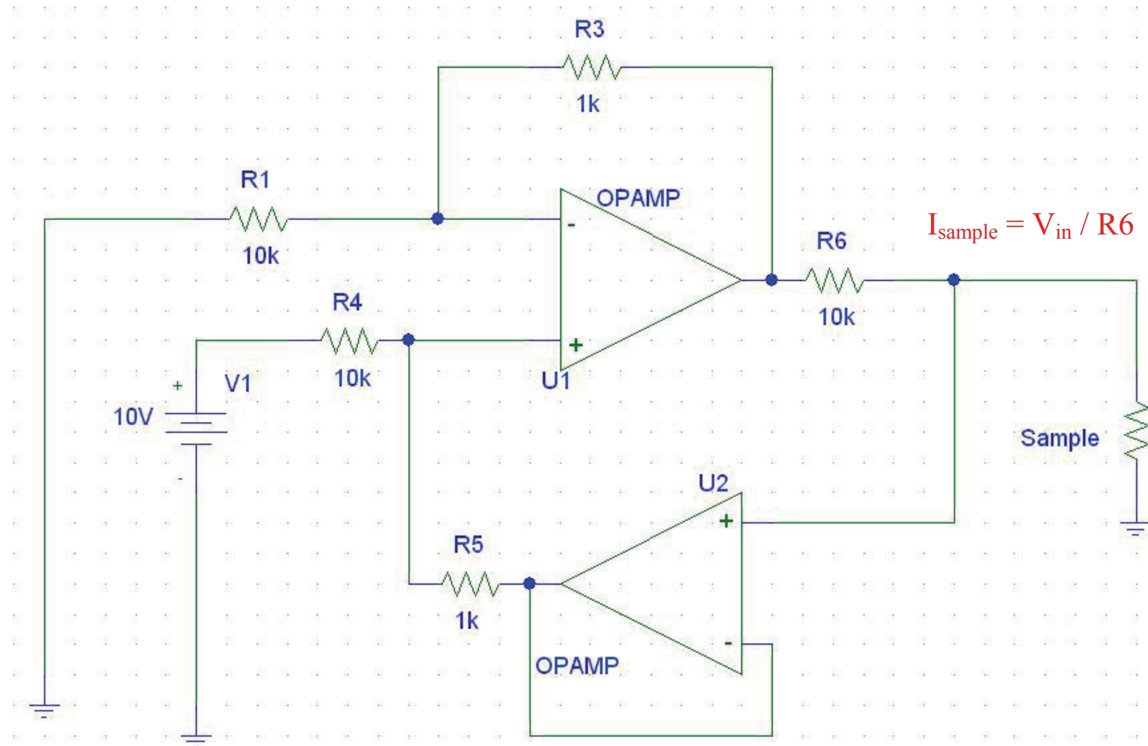

FIGURE A.2. Voltage controlled variable current source

In the pH-stat system, pH meter acting as a feedback detector, measures pH of sample and sends the value to the LabVIEW program at the sampling frequency of 1 Hz. A micro pH electrode is used in order to measure pH of a sample at the scale of microliter. The pH meter is connected to computer via RS-232 interface. It is observed that due to the lack of isolation at the communication port of pH meter, electrical cross-talk between DAQ Pad and pH meter can occur. Therefore, a RS-232 optical isolator is needed between computer and the pH meter to eliminate the common ground voltage bias which may result in a significant measurement error. Figure A.3 shows the RS-232 optical isolator used in our system (Model 9POP4, B&B Electronics Inc., Ottawa, IL, USA).

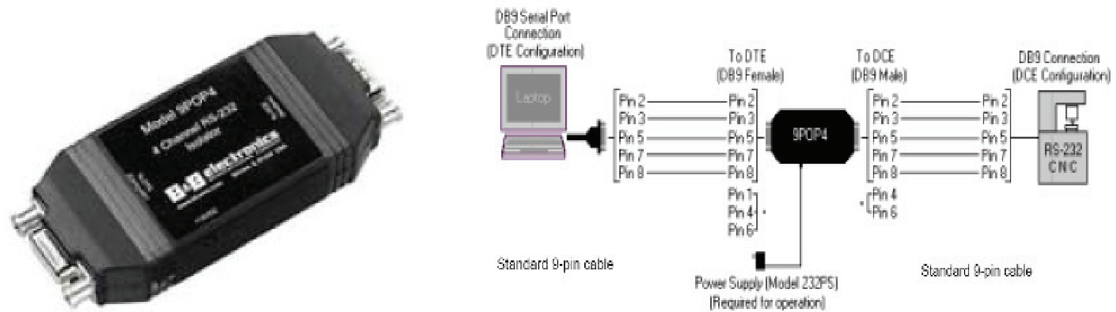

FIGURE A.3. RS-232 optical-isolator (picture from B&B Electronics Inc., Ottawa, IL, USA)

A-I.3. PID control algorithm

LabVIEW program is the core component of this system. It contains the several important functions including communication between different hardware such as pH meter and DAQ pad, the feedback control algorithm for pH-stating, and user interface for graphics plotting and parameter manipulation. The pH value of the sample is continuously sensed from the pH meter and simultaneously compared with the pre-set pH. Then, the required output voltage for counter-balancing pH variation is calculated according to PID algorithm. The voltage is sent to DAQ pad and converted by the voltage-to-current circuit. Consequently, a current is fed to the sample to cause an acidic or basic shift in pH of the sample. Thus, all the components are bridged and controlled via LabVIEW and the PID algorithm encoded in LabVIEW. The preliminary LabVIEW program with PID controller is shown in Figure A.4 and its performance of pH manipulation in microliter droplets is shown in Figure A.5.

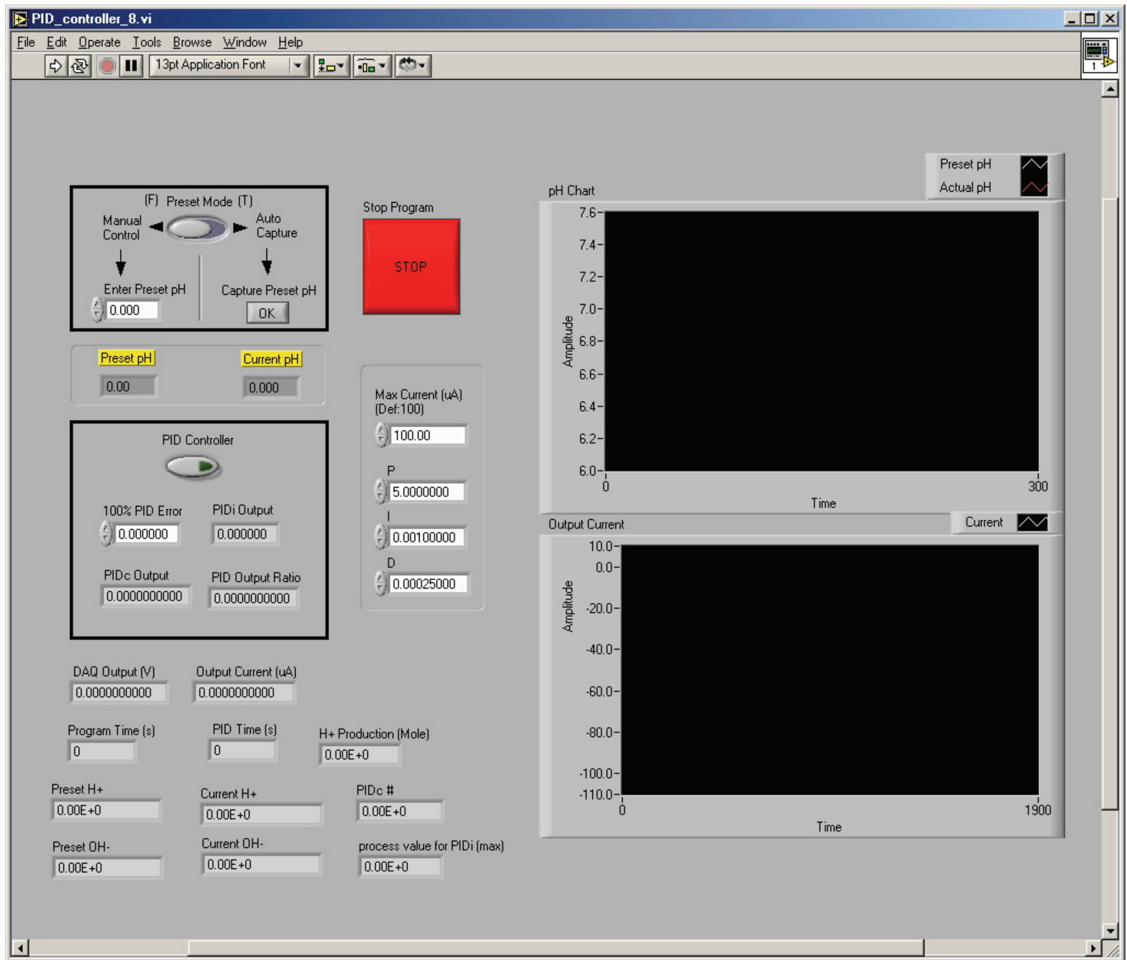

FIGURE A.4. The first version of LabVIEW controller. It contains the basic PID control algorithm and user interface. The graphic panel on the top-right shows the real-time pH and pre-set pH over time and the panel on the bottom-right shows the output of current.

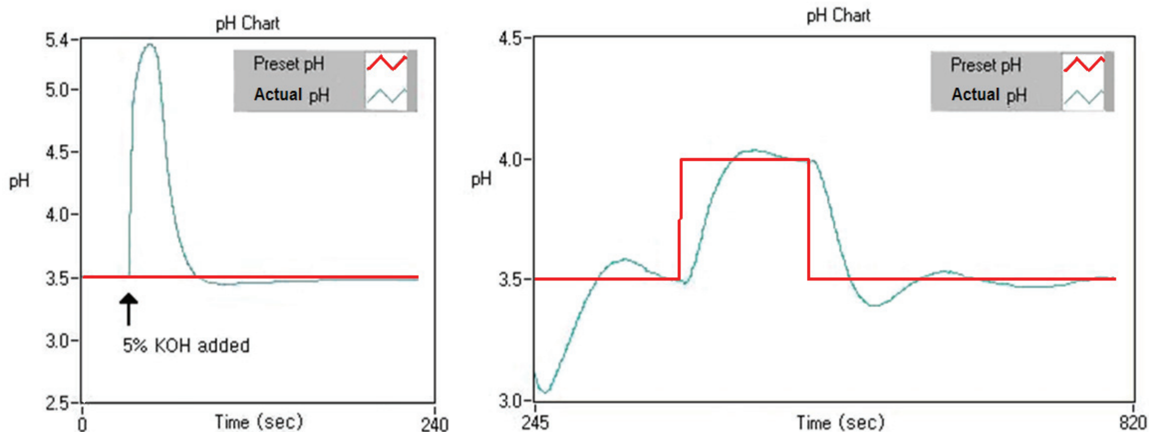

FIGURE A.5. Performance of PID controller. (a) External perturbation from 5% KOH solution on the tip of a needle (~0.5 μL). The pH of the sample (0.1M potassium nitrate) was restored to the original pre-set value within one minute. (b) Sample set pH manipulation. The pH of the PBS sample tracked the changes in the pre-set pH value.

A-I.4. Chamber for pH-stat on the RSS

Samples used in the Rotating Sample System (RSS) are of high surface-to-volume ratio. In order to minimize sample evaporation and the disturbance from CO_2 in the atmosphere, an acrylic chamber with DI water channels surrounding the RSS cell (Figure A.6) was built to keep the cell in a humidified and isolated environment. A small hole was made to equilibrate the air pressure with the outside atmosphere. The chamber also provides for positioning of the combination pH electrode and the two anti-parallel air nozzles. The speed of air flow is monitored by volumetric flow meters (Cole-Parmer Instrument Co., Niles, IL, USA).

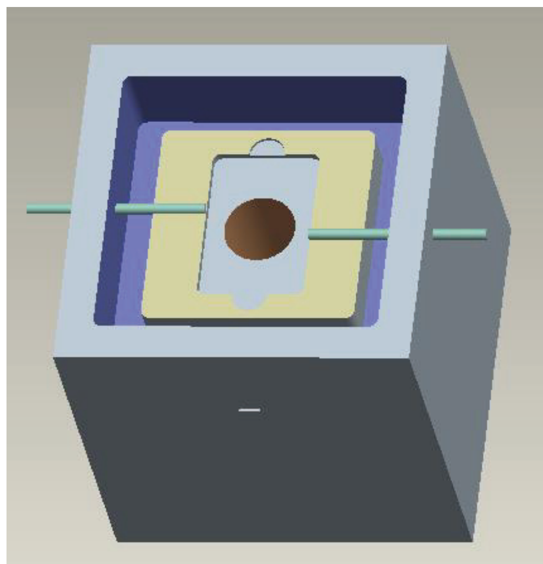

FIGURE A.6. Acrylic chamber with DI water channels surrounding the RSS cell and air jets housing. The RSS cell is placed in the rectangular dented surface whose depth is equal to the thickness of the cell substrate. The hole in the center underneath the RSS cell is the lower compartment containing the gold counter electrode in the same supporting electrolyte solution as the sample. Housing support for the micro pH electrode is on the cap (not shown).

A-I.5. Elimination of CO_2 interference

As mentioned previously, an acrylic chamber with DI water channels surrounding the RSS cell was built to keep the cell in a humidified and isolated environment. A small hole was made to equilibrate the air pressure with the outside atmosphere. Even in this isolated environment, however, atmospheric CO_2 from the air jets used for sample rotation can still noticeably acidify unbuffered samples (Figure A.7, red curve). Therefore, a modification for eliminating CO_2 from the air flow has been developed. The air flow was driven through a porous air stone to produce fine air bubbles with a large total surface area that pass through saturated $Ca(OH)_2$ solution with a long pathway for both humidification and CO_2 elimination. Saturated $Ca(OH)_2$ solution absorbs CO_2 forming $CaCO_3$ precipitation. This setup modification has been optimized and tested. We found that it successfully eliminated most CO_2 from the air jets and thus, reduced the background pH drift to less than 0.1 pH unit for 4 minute period of sample rotation. (Figure A.7, black curve). Effecting convective mixing of the $Ca(OH)_2$ solution and with perfected chamber sealing the pH drift could be further reduced to 0.03 for 4 minutes (Figure A.7, blue curve) (Kao, Hsu et al. 2008).

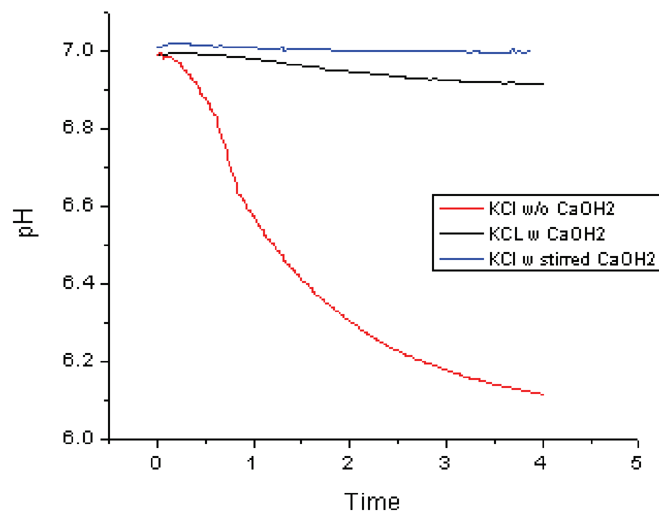

FIGURE A.7. Sample pH drift due to CO_2 influx. Red: sample (0.1 M KCl) is rotated by air flow humidified by DI water. Black: sample is rotated by air flow humidified by $Ca(OH)_2$ solution. Blue: sample is rotated by air flow humidified by stirred $Ca(OH)_2$ solution and with perfected chamber sealing.

A-I.6. Minimizing the effect of protein adsorption at the liquid-air interface

Proteins increase viscosity and therefore reduce diffusion rates. This will have an effect on electrochemistry in biological fluids. Proteins are also surface active and will therefore partition into the air-liquid interface. This may adversely affect the coupling between air jet and sample drop, and ultimately, the hydrodynamic performance of the RSS. Previous work from our laboratory has demonstrated that protein-protein interaction in a biological sample substantially alter surface tension of the sample and as a consequence the droplet will not be rotated efficiently by the air jets (Shetty 2005).

Protein adsorption at the liquid-air interface hence becomes a major problem because the forced convection for homogenization of pH in the solution is important for pH-stating.

According to the discovery made in our laboratory addition of diluted lipid will disrupt the protein film effectively such that the speed of rotation could be restored (Shetty 2005). Though we do not fully understand the mechanism of how the protein/lipid interaction can disrupt the protein film, it is an interesting observation. Intralipid 20% (a 20% lipid fat emulsion containing all the biologically essential fatty acids as a model lipid) was diluted 1:100 in sodium chloride solution. The lipid solution was then mixed with the enzyme preparation to reduce the effect of protein-protein interaction and improve forced convection in RSS (Figure A.8) (Kao, Hsu et al. 2008).

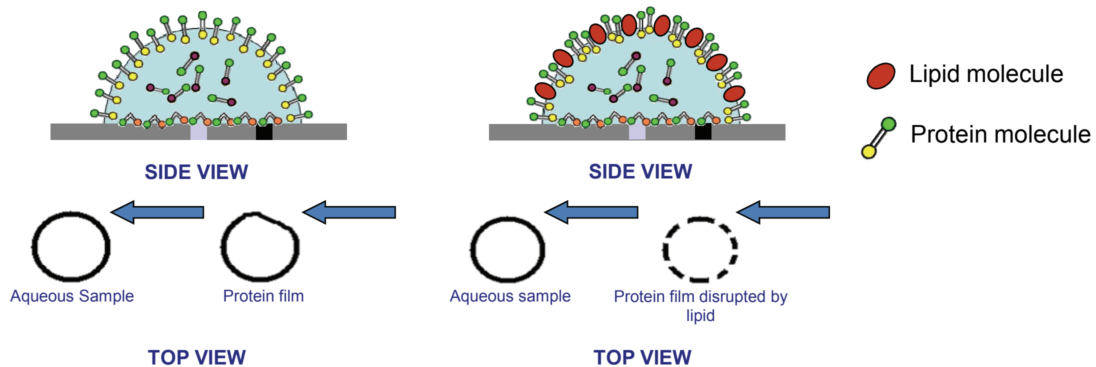

FIGURE A.8. Protein adsorption in RSS sample: (a) Protein aggregation at liquid-solid and liquid-air interface (b) Lipid addition disrupts protein-protein interaction at liquid-air interface and restores the rotation speed of RSS.

A-I.7. Micro pH electrode

After exploring the commercially available pH electrodes for microliter-scaled samples, we have tested two micro pH electrodes for our system: the solid-state based micro pH electrode PHR-146B (Lazar Research Inc., Los Angeles, CA, USA) and the glass micro pH electrode MI-4154 (Figure A.9a) (Microelectrodes Inc., Bedford, NH, USA). We note that the performance of PHR-146B cannot meet our expectation when it was utilized for actual enzyme experiments. The main reason is that pH sensing of a solid state electrode is less stable and the response time is slower than a glass pH electrode. Therefore, we have adapted the MI-4154 electrode (Figure 1a) which has only 1 mm tip diameter and can be connected to a Fisher AR-15 digital pH meter for our pH-stat system.

The MI-4154 glass pH electrode presents several advantages over the PHR-146B solid state pH electrode. First of all, it offers faster pH response and more stable pH readings (Figure 1b), which are critical characteristics for accurate PID feedback control. Secondly, the glass membrane is easier to clean especially when exposed to biological samples. Finally, the PHR-146B contains a battery-powered circuitry to amplify the signal output but it is poorly designed as the built-in battery is not replaceable. Therefore, it is necessary to purchase a new electrode every 12 months or so when the battery runs out. The glass pH electrode, on the other hand, does not require external power for measurement hence it can last many years if handled with care.

pH electrode handling and cleaning: Although the glass pH electrode is better suited for this project, this tiny glass electrode is quite fragile. Any contact during the experiments can damage the electrode. Therefore, we have implemented a micro

manipulator (M3301, World Precision Instruments Inc, Sarasota, FL, USA), as well as fabricated a customized electrode holder and electrode protector to protect the electrode body of MI-4154. Electrode cleaning after exposed to protein/biological samples is by soaking in a cleanser "Tergazyme", an enzyme active powdered detergent (Alconox Inc, White Plains, NY, USA) for 5 minutes and then rinsed with DI water.

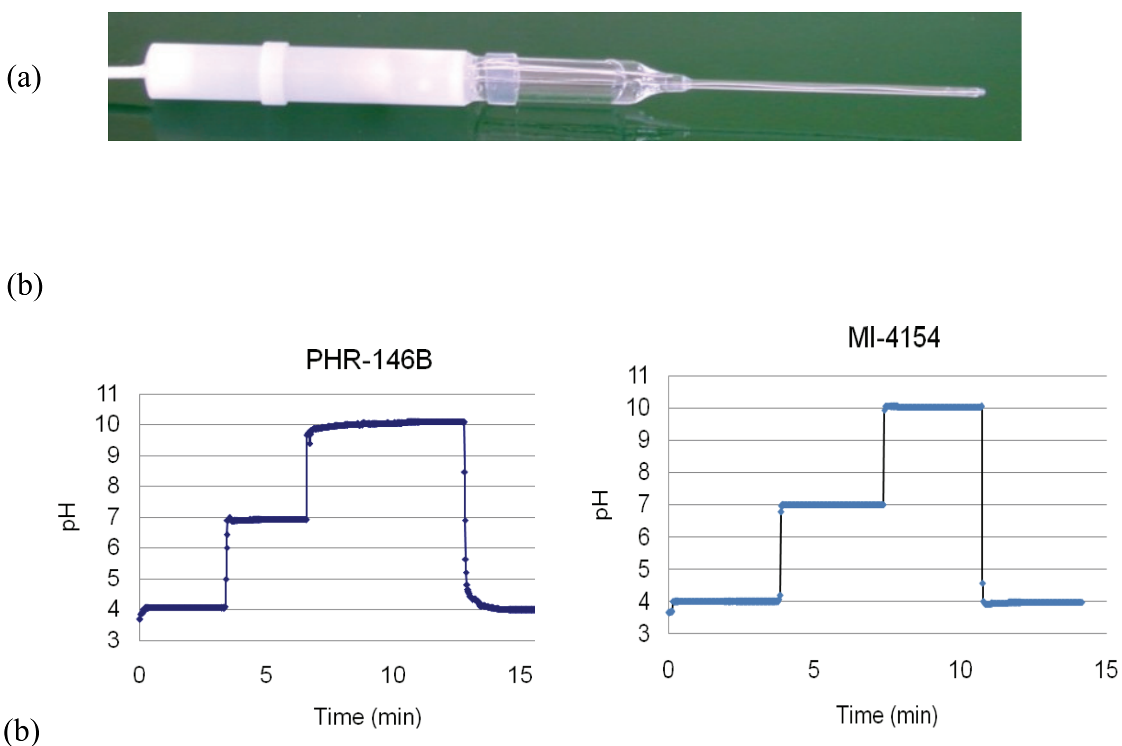

FIGURE A.9. Micro pH electrode. (a) A glass micro pH electrode (MI-4154) has been implemented (picture from Microelectrodes Inc). (b) PHR-146B and MI-4154 are tested in bulk pH buffer (pH 4, 7 , 10) by quickly changing from pH 4 to 7, 7 to 10, then back to 4. The results indicate that MI-4154 offers more stable pH readings and faster response time compared to PHR-146B.

A-I.8. Implementation of the advanced PID control

General PID controller consists of three adjustable parameters (k_P, k_I, k_D) which stand for the gain of proportional, integral, and differential errors (A.1). It is known a basic PID control algorithm with adjustable (k_P, k_I, k_D) parameters is not ideal for non-linear control. The output of a PID controller ($u(t)$) is a combination of proportional ($u_P(t)$), derivative ($u_D(t)$), and integral ($u_I(t)$) outputs. To obtain stable pH-stating, implementing an enhanced integral output ($u_I(t)$ is critical to counterbalance the pH drift caused by enzyme activity at the pH-stating point. The reason is that when the pH converges very close to the set value, both the proportional ($u_P(t)$) and derivative ($u_D(t)$) outputs become virtually zero (A.1). On the other hand, while the pH is farther away from set value the weight of the integral component (k_I) needs to be much smaller than that of the proportional (k_P) and derivative (k_D) elements otherwise the integral term will cause saturation of the current output and thus may cause a significant overshoot in the other pH direction. Therefore, an appropriate PID control algorithm for this application requires a non-linear integral output ($u_I(t)$) which would be increased or decreased according to the error in pH is smaller or larger, respectively. We have implemented and optimized an advanced PID controller by which the non-linear integral output is adjusted automatically within the set point range (A.2).

$$u(t) = k_P e + k_I \int_0^t e dt + k_D \frac{de}{dt} \tag{A.1}$$

$$u_I(k) = k_I \sum_{i=1}^{k} \left[\frac{e(i)+e(i-1)}{2} \right] \Delta t \left[\frac{1}{1+\frac{10e(i)^2}{SP_{rng}^2}} \right] \quad \text{(A.2)}$$

where u_I is integral action, k_I is integral parameter, e is error between set value and actual value, SP_{rng} is the set point range.

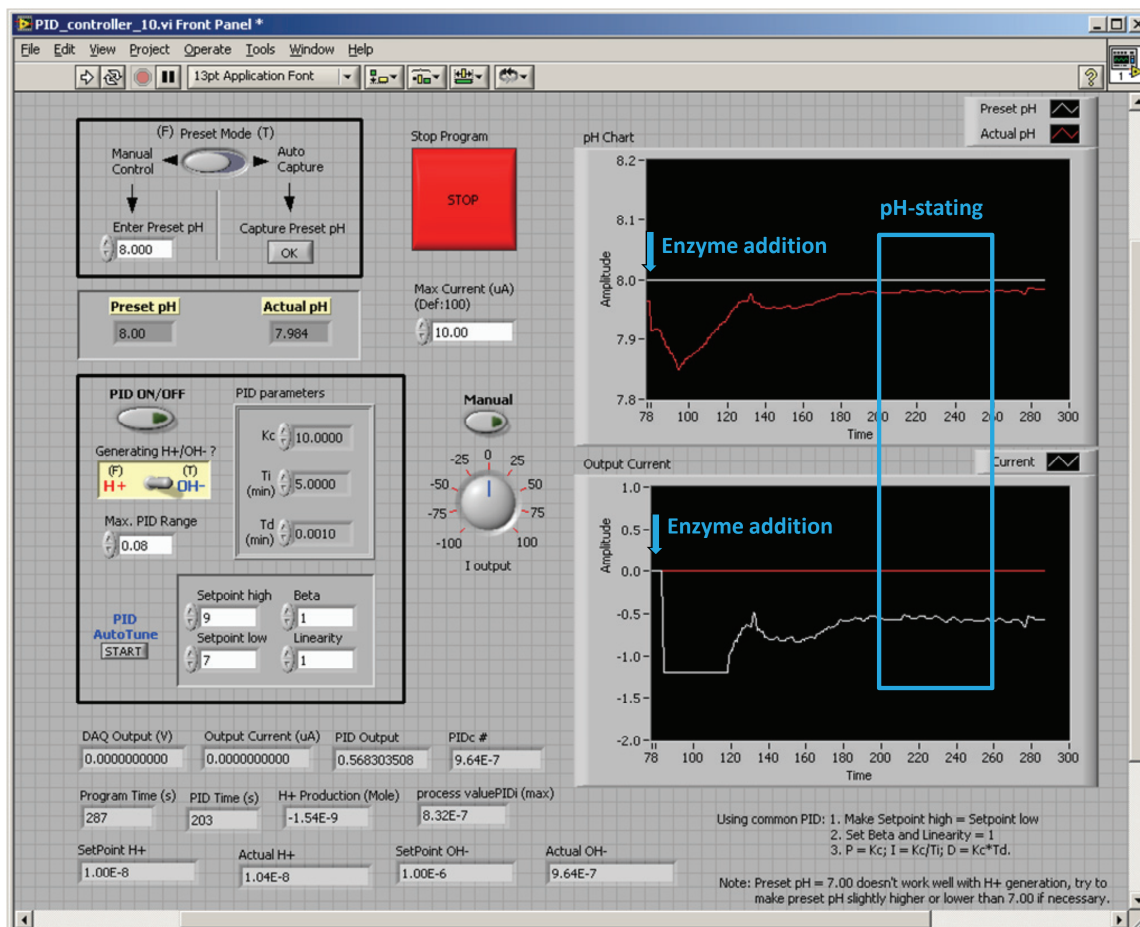

FIGURE A.10. Typical operation of the pH-stating assay for enzyme kinetics. Substrate and enzyme solutions are mixed when pH reading of the sample is stable. The enzyme catalytic reaction shifts the pH and the output current calculated by the advanced PID controller would counterbalance the pH shift and bring the pH to its set value. Once the steady-state has reached, average current in an approximately one-minute pH-stating window will be used for calculation of enzyme kinetics.

A-I.9. Reference

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Appendix II. Possible errors during pH-stating and the trouble-shooting procedures

A-II.1. Background pH drift

Prior to perform pH-stating a test of background pH drift of a rotated sample should be checked. When the background drift is significant, the possible causes can be:

1) Acid/base shift due to air flow: The air flow for sample rotation passes through the humidification solution and equilibrates its CO_2 content with the solution. Once the humidified air is in contact with the sample, owing to the large surface area relative to the small sample volume and the eventual difference in CO_2 contents, absorption/desorption of CO_2 into/from sample may occur and that causes the sample pH to be "titrated" by the air flow, especially in non-buffered samples. In order to keep the pH baseline stable during the pure sample rotation, it is suggested to adjust the pH buffer of the humidification solution close to, but a little higher than the desired pH value for pH-stating.

2) Junction titration: Diffusional pH titration from the liquid junction is another possibility to cause background pH drift (Hui and Gratzl 1997). However, it can be easily mitigated by adding 10 mM phosphate buffer in the liquid junction.

3) Residual enzyme: Residual enzyme on the glass substrate, pH electrode, or in the contaminated tips of transfer pipette can react with substrate sample and result in background pH drift. Use disposable pipette tips and enzyme active detergent or alcohol to clean the glass substrate and pH electrode thoroughly can eliminate this problem.

A-II.2. Erratic pH-stating

Potential factors causing unsteady pH-stating are:

1) Insufficient rotating speed: Ineffective stirring may result in non-uniform pH distribution in the sample that misleads the feedback controller to generate incorrect current output. Adding a tiny amount of dye into a test sample one can visualize how fast the dye is being mixed and that would help determine whether or not the erratic pH-stating is due to insufficient rotation. If confirmed, several things need to be checked in order to restore proper convection: (1) make sure the air jets are located properly and the air flow is tangential to the droplet; (2) check the integrity and the shape of the droplet; (3) make sure the air flow rate is accurate by monitoring the volumetric flow meters (Cole-Parmer Instrument Co., Niles, IL, USA); (4) if the low hydrodynamic efficiency is caused by protein adsorption at the liquid-air interface, applying diluted lipid into the protein solution should improve convection.

2) Unstable pH reading: If the pH reading is jittery, that can be due to electrical interference. If the pH response is slow, then it may due to contamination of the pH sensing membrane or the junction of pH electrode.

3) Improper PID parameters: The typical *P*, *I*, *D* gains in the LabVIEW program are set to be (10, 2, 0.001) for AChE assay. Parameters such as setpoint high and setpoint low for advanced PID control are assigned to be ±1 pH unit of the desired pH. It is important to keep the *I* value low so that the accumulated error is not over-weighted. In addition, a lowered *D* value helps reduce jitteriness of the current output. In most cases, fine-tuning controller parameters such as "Max. PID Range" and "Max current" can correct the controller performance for achieving stable pH-stating.

A-II.3. Reference

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