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Short communication

A quantitative electrochemical assay for liver injury

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ABSTRACT

Liver diseases represent a vastly underestimated and historically neglected public health problem, disproportionately affecting those in low- and middle- income countries (LMICs). Patients on hepatotoxic medications, such as HIV and TB medications, need consistent monitoring of liver function as part of their standard of care. In high resource settings, this is often the case, but in LMICs traditional methods fail due to high cost and lack of proper equipment, supplies and trained personnel. To address this gap in technology and patient care, we have developed a quantitative, electrochemical assay capable of quantifying levels of alanine aminotransferase (ALT), a primary biomarker associated with liver function. We can quantify ALT with increased sensitivity (1.53 nA/(U/L*mm^2) and over a wide, linear concentration range (40–1990 U/L). The assay demonstrated in this study can be used to overcome several pressing challenges associated with effective, timely treatment of liver disease in LMICs.

1. Introduction

Liver disease represents a significant public health burden in both high- and low-income countries, causing over two-million annual, global deaths (Byass, 2014; Marcellin and Kutala, 2018). The vast majority of individuals affected live in low- and middle-income countries (LMICs) in Africa and Asia, suffering the disproportionate brunt of the impact (Byass, 2014). In Africa alone, about 100 million individuals are infected with chronic viral hepatitis B or C, while this number is more than 75% less in high-income countries (Cainelli, 2012). Acute liver failure (ALF) - or loss of hepatocyte function without evidence of chronic liver disease - is caused by certain strains of viral hepatitis as well as the excessive use of medications, specifically acetaminophen, anti-tuberculosis and anti-retroviral therapies (ART) (Lee, 2013; Wondemagegn et al., n.d.). In high-income settings, the ALF survival rate without liver transplantation is as high as 40%, but this survival rate falls drastically in low-income settings where cases are diagnosed too late or considered untreatable (Cainelli et al., 2016).

Despite the significant mortality burden, liver diseases are historically neglected due to a lack of public health surveillance and reporting systems, as well as a lack of national and international programs targeting these diseases (Cainelli, 2012; Marcellin and Kutala, 2018). A large portion of deaths due to liver diseases can be cured (e.g. chronic hepatitis C), treated (e.g. chronic hepatitis B) or prevented (e.g. acute liver failure due to medications) if prompt diagnosis is made. However, there is a lack of robust, easy to use, and reliable methods for early diagnosis to manage and treat liver failures in low-income settings (Marcellin and Kutala, 2018).

Furthermore, in countries with a high burden of HIV and TB co-morbidity, 11% of adults on anti-TB medications and 14–20% of adults on ART medications show elevated serum liver enzymes as a marker of hepatocellular injury (Ramappa and Aithal, 2013; Wondemagegn et al., n.d.). For these patients, liver complications can lead to severe liver failure and death. Therefore, these patients require consistent screening of liver-function as part of standard of care. Unfortunately, for HIV and TB positive patients with limited resources, the need for regular screening cannot be met due to cost and logistics of available diagnostics that require extensive human, infrastructural and capital resources. Therefore, the need for low-cost, easy to use, and reliable diagnosis of ALF is critical for these patients, particularly those who need regular, consistent monitoring of liver function.

1.1. Current liver-screening technology and associated challenges

Currently, the standard of care for liver function testing involves monitoring alanine aminotransferase (ALT), a primary biomarker associated with liver damage. ALT, quantified in units/liter (U/L) (Supplemental Fig. 1) is typically monitored through a venipuncture blood draw, followed by centrifugation to separate serum or plasma, and testing of serum or plasma through an absorbance-based assay on a

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large, automated platform. Typically, clinical assessment also includes measurement of aspartate aminotransferase (AST), an enzyme similar in function to ALT that is also found in the liver. Although AST levels provide more information as to the source of disease, ALT levels on their own are sufficiently indicative of liver damage. Currently, these gold-standard absorbance-based tests are not often used in low-resource settings due to high cost, and lack of equipment, supplies, or trained personnel (Byass, 2014; Marcellin and Kutala, 2018).

Prior studies have reported detection techniques for measurement of ALT from a fingerstick of whole blood (Compagnone et al., 1992; Cooper et al., 1991; Han et al., 2011; Kihara et al., 1984a, 1984b; Matsuzawa and Katunuma, 1966; Mizutani et al., 1998; Moser et al., 1997; Peguin et al., 1989; Pollock et al., 2012; Xuan et al., 2003). The methods used include colorimetric, spectrophotometric, electrochemical and radiochemical techniques.

More recently, electrochemistry has been the focus of many developments in the field (Chen et al., 2018, 2014, 2011), with many groups working on ALT detection moving almost exclusively towards electrochemical detection techniques due to the increased sensitivity of measurements (Chang et al., 2007; Jamal et al., 2009; la Cour et al., 2016; Paraíso et al., 2014; Thuy et al., 2016). Many of these groups use platinum or palladium modified working electrodes coupled to a secondary detection enzyme, demonstrating assay sensitivity in the pico to nano range. These sensors all vary in their specifications, trading off between improvements in sensitivity, limit of detection, linear range, response time, sample volume, and fabrication complexity.

Despite these advancements in assay development, cost-effective, quantitative, and reliable detection of liver enzyme levels still poses a major challenge in low- and middle-income countries. To address this gap, we have demonstrated the use of a novel electrochemical assay, capable of quantitatively measuring levels of ALT with an increased sensitivity over previously published studies and over a wider range of ALT concentrations (Chang et al., 2007; Compagnone et al., 1992; Han et al., 2011; Jamal et al., 2009; Kihara et al., 1984a, 1984b; Paraíso et al., 2014; Peguin et al., 1989; Song et al., 2007; Thuy et al., 2016; Upadhyay et al., 2006). This assay has the potential to be used at the point of care, as it can be engineered to be low-cost, self-contained and user-friendly. With prompt quantification of liver enzymes at the bedside, healthcare workers could benefit from this system, making rapid and necessary medical decisions to save patient lives.

2. Materials and methods

2.1. Assay design

This assay is based on our ability to measure ALT levels in whole blood by leveraging the enzymatic activity and monitoring the depletion of alpha-ketoglutarate, in the presence of the appropriate starting reagents. Hydrogen peroxide (H_2O_2) is used to quantify alpha-ketoglutarate as a proxy for ALT concentration, as shown in Fig. 1.

ALT catalyzes the transamination of L-alanine and alpha-ketoglutarate to pyruvate and glutamate. In a subsequent reaction, leftover alpha-ketoglutarate can react with hydrogen peroxide, depleting the concentration of hydrogen peroxide proportionally to the amount of alpha-ketoglutarate left in solution (Long and Halliwell, 2011). Hydrogen peroxide is then coupled to an electron transfer mediator, Prussian Blue (PB), and detected amperometrically. Amperometry is a strong yet simple technique for electrochemical detection, and one that can be converted into a miniaturized test without compromising sensitivity (Wu and Zaman, 2015). Using commercially available glucometers as detection units, amperometry has been successfully demonstrated in the context of glucose, lactose, cholesterol, and alcohol quantification (Cardosi and Liu, 2012; Wu and Zaman, 2015). This study extends prior studies to detect levels of ALT in solution at physiologic and pathologically relevant levels.

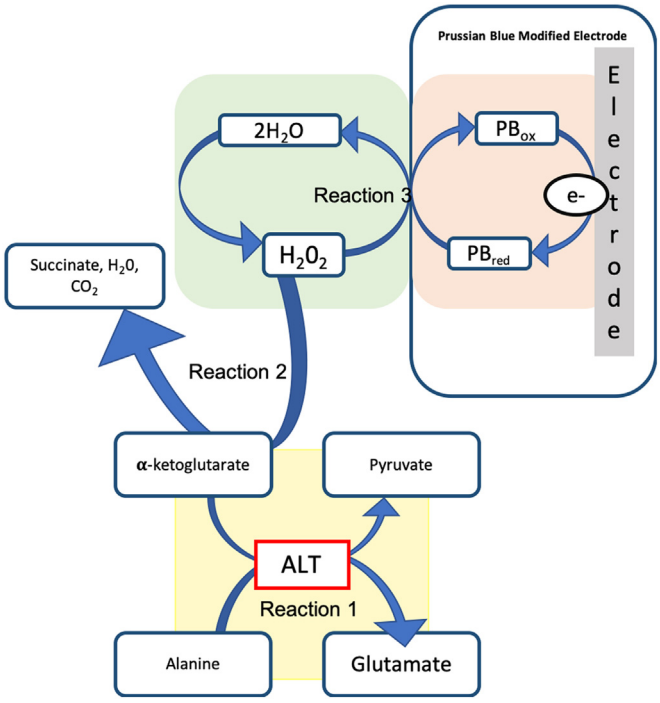

Fig. 1. Assay Schematic. Reaction 1, 2, and 3 occur in series. In the first reaction, ALT catalyzes a reaction consuming alpha-ketoglutarate. In reaction 2, leftover alpha-ketoglutarate reacts with hydrogen peroxide, reducing the amount of peroxide in solution (Long and Halliwell, 2011). In reaction 3, the remaining peroxide is detected at Prussian Blue modified electrodes. Overall, an increase in current is indicative of an increase in ALT concentrations; at higher ALT levels, more alpha-ketoglutarate is consumed, leaving less to react with peroxide and more peroxide to react at the electrode.

2.2. Materials and reagents

Alanine, alpha-ketoglutarate, pyridoxal phosphate, hydrogen peroxide, sodium hydroxide, and tris hydrochloride (Tris) were acquired from Sigma. Alanine aminotransferase from bovine liver was acquired from Lee BioSolutions.

2.3. Experimental protocols

All samples were made in 0.5 M tris buffer, pH 8.5. The pH of the buffer was adjusted with sodium hydroxide. The final reagent concentrations were 200 mM Alanine, 15 mM Alpha-ketoglutarate, and 10 mM pyridoxal phosphate (PLP). 30% hydrogen peroxide was diluted with tris buffer at a ratio of 50:950 µL. ALT concentrations varied throughout experiments, spanning the range of physiologically relevant levels; these concentrations are noted appropriately. At all concentrations, ALT was added to the reaction mixture in 50 µL of solution. This was done to simulate a 50 µL "blood spot" that could come from a fingerpick. Therefore, all dilutions that would be done to blood are done in this protocol and no additional dilution steps would need to be taken when using this protocol with blood samples.

Coupled reactions for electrochemistry experiments occur in 4 phases in the following order: 1) Incubation of ALT (50uL sample containing a range of levels), alanine, and pyridoxal phosphate at 60 °C for 30 min, 2) Addition of alpha-ketoglutarate, incubation at 60 °C for 40 min, 3) Addition of hydrogen peroxide, incubation at room temperature for 30 min.

2.4. Electrochemical testing and analysis

Screen printed electrodes (SPEs) were acquired from DropSens

(DRP-710) and contained a Prussian- Blue modified carbon electrode as the working, carbon as the counter, and silver as the reference electrode. Amperometry was performed with a commercial potentiostat (ChI 1200B) at − 0.1 V versus carbon and resulting current was measured for 1s. 50 µL of solution (from ~150uL total solution) was applied to the electrode and readings were taken immediately at room temperature.

An average current over the last 3 data points of each trace was calculated: this value was then used as the signal for that trace. For peroxide studies, delta current is defined as the current due to tris buffer subtracted from the current signal at varying peroxide levels. For ALT/AST studies, delta current was defined as the current at 40 U/L ALT (physiological baseline) subtracted from the current signal at elevated levels of ALT. Data is presented as the mean ± the standard error over 5 measurements.

3. Results

To test the efficacy and feasibility of our assay, we first measured the hydrogen peroxide response on PB mediated electrodes. 50 µL samples of varying peroxide concentrations were pipetted onto the electrodes and detected via amperometry at − 0.1 V versus carbon. The measured current increased with increasing hydrogen peroxide concentration, as shown in Fig. 1a. At lower concentrations of peroxide (< 10 mM) a linear increase in current was observed. This trend was exploited for assay development.

The coupled reactions, performed using the protocol outlined above, were used to produce a repeatable ALT response curve, as shown in Fig. 2b. ALT-coupled peroxide reactions produced current that increased linearly with increasing ALT concentration and encompassed a wide concentration range. Because of the linear relationship, current can be used as a reliable indicator of ALT concentration. Cross reactivity was also tested using AST, Fig. 2b, and no pattern in signal was detected, showing that our assay is specific to ALT. To validate the resolution of our assay at lower ALT concentrations, further testing with coupled reactions was performed between 40 and 1000 U/L ALT, as shown in Fig. 3. In this range, current from the coupled-reactions still increased linearly with increasing ALT concentration, and therefore poses as a reliable indicator for ALT concentration if averaged over many tests.

ALT coupled to hydrogen peroxide produces current proportional to ALT concentration, encompassing a wide range of ALT concentrations (40 UL – 1990 U/L) with an R^2 value of 0.999. This methodology proved promising, but assay resolution at low ALT levels is of particular importance, as patients with only mildly elevated ALT are usually asymptomatic and often the most difficult to diagnose (Johnston, 1999). Even at low ALT concentrations, the current signal remained linear, with an R^2 value of 0.994. Based on this linear signal, current values can be used as an indicator of ALT concentrations in solution. The detection limit and sensitivity are found to be 20.6 U/L and 1.53 nA/(U/L*mm^2), respectively.

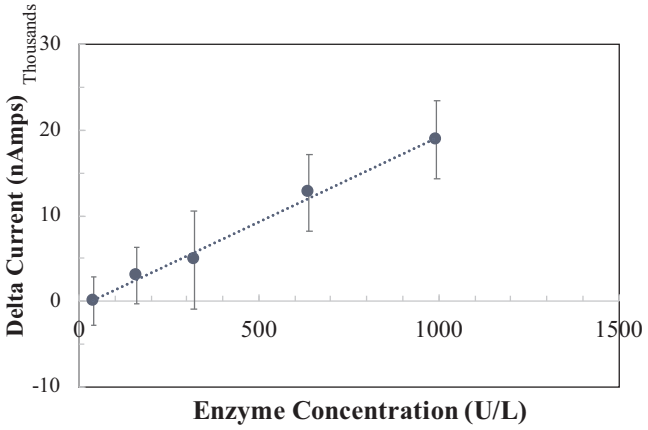

Fig. 3. Amperometric detection of ALT-coupled reactions at − 0.1 V versus carbon.

Additionally, this signal is specific to ALT, as shown through cross reactivity testing with AST. The data presented here has established a proof-of-concept and will allow us to move forward with device design to create an integrated system capable of performing ALT tests at the point of care.

4. Discussion

The standard-of-care for ALT testing in high-income settings consists of a venipuncture blood draw, followed by centrifugation to separate serum or plasma, and testing of serum or plasma through an absorbance-based assay on a large, automated platform. In low-income settings, this type of testing is often not feasible due to high associated costs, and lack of equipment, supplies and trained personnel. Even when the resources for standard of care testing are available, the need to send ALT tests to a central laboratory delays the process immensely. Oftentimes, tubes get lost in transition to and from the central laboratory; when results are returned, weeks or months have often past.

Due to the clinical importance of monitoring ALT in patients with liver diseases, many research groups have developed detection methods for this purpose. These technologies range from colorimetric, spectrophotometric, chemiluminescent, and fluorescent detection methods to

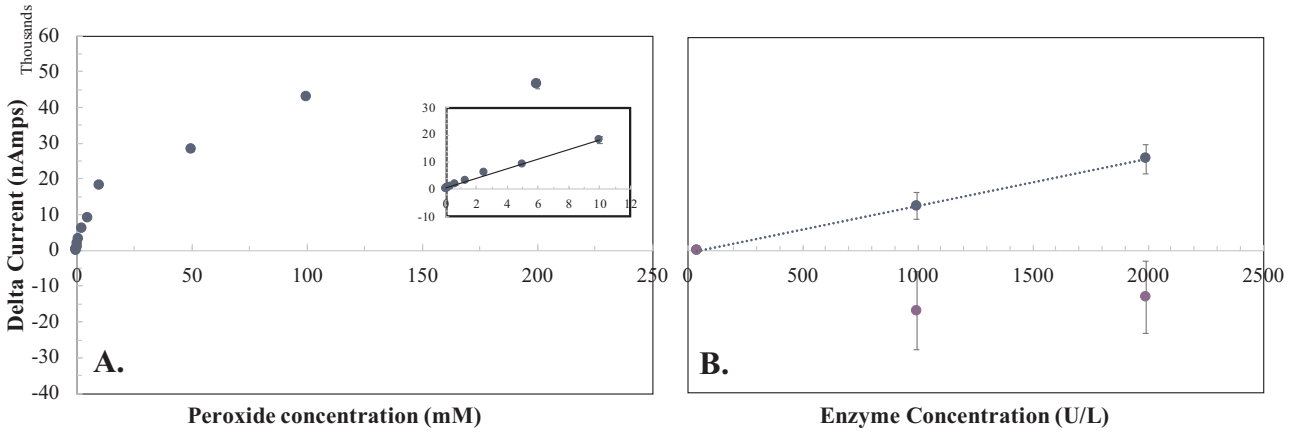

Fig. 2. Amperometric detection of Peroxide (A) and ALT-coupled reactions (B) at − 0.1 V versus carbon. A). Blue dots represent current measurements due to peroxide solutions. B). Blue dots represent current measurements due to the assay run with ALT. Purple dots (negative) represent current measurements during cross reactivity testing done with AST as a negative control.

radiochemical and electrochemical techniques. Colorimetric assays for ALT date back to the 1960s, with Matsuzawa and Katunama reporting a method for ALT detection involving coupling enzymes and diazonium salt (Matsuzawa and Katunuma, 1966). Their assay was rapid, accurate, and produced color according to extent of ALT activity. A spectrophotometer was used to measure optical density and ALT concentration was calculated based on a concentration curve. For this assay, the need for a spectrophotometer prohibits use at the POC. In a more recent example, Pollock and Rolland et al. developed a paper-based point of care assay for semiquantitative measurement of alanine aminotransferase from a fingerstick of whole blood. The device has a visual colorimetric readout, grouping ALT into three ranges, $> 5\times$ upper limit of normal (ULN), 3–5× ULN, and $< 3\times$ ULN (Pollock et al., 2012). This semi-quantitative liver function test is in the process of submission for regulatory approval for point-of-care use. Although this device overcomes a lot of barriers in liver function testing at the point of care, it still lacks the ability to deliver quantitative clinical information— information extremely important in diagnosing the source of the liver dysfunction and making relevant clinical decisions (Kihara et al., 1984a, 1984b; Matsuzawa and Katunuma, 1966; Peguin et al., 1989).

Recently, ALT detection via electrochemical assays have been the focus of many research groups due to the high sensitivity which comes from electrochemical methodology (Chang et al., 2007; Compagnone et al., 1992; Cooper et al., 1991; Han et al., 2011; Kihara et al., 1984a, 1984b; la Cour et al., 2016; Mizutani et al., 1998; Peguin et al., 1989; Thuy et al., 2016; Xuan et al., 2003). Most of these assays use ALT coupled to either a pyruvate or glutamate oxidase with subsequent detection of H_20_2 at a modified electrode. For example, Paraiso et al. developed a bioelectrode for detection of ALT using pyruvate oxidase in a coupled reaction and poly(4-aminophenol) and 4-aminoantypyrine as mediators (Paraíso et al., 2014). These electrodes showed a low detection limit of $2.68 * 10^{-5}$ U/L ALT but were fabricated using electropolymerization techniques (requiring many time-consuming preparation steps such as washing, ultrasonicating, and deaerating) and needed low temperature storage conditions (8 °C). Additionally, Thuy et al. developed a micro-platinum wire biosensor modified with glutamate oxidase as a coupled enzyme and polymer layers, polypyrrole and Nafion, as mediators (Thuy et al., 2016). This group showed a detection limit of 8.48 U/L ALT and a sensitivity of 0.059 nAmps/(U/L*mm^2). Preparation and maintenance of these electrodes was quite intensive, requiring many dip-coating (~90) and baking steps as well as storage at − 20 °C.

Compared to ALT electrochemical assays from other research groups, our assay shows increased sensitivity (up to ~10^5 fold) (Chang et al., 2007; He and Chen, 1997; Jamal et al., 2009; Kihara et al., 1984a, 1984b; Thuy et al., 2016) and a wider linear range (Chang et al., 2007; Compagnone et al., 1992; Cooper et al., 1991; He and Chen, 1997; Jamal et al., 2009; Kihara et al., 1984a, 1984b; Paraíso et al., 2014; Song et al., 2007; Thuy et al., 2016; Upadhyay et al., 2006) than many. The lower limit of detection (20.6 U/L) shown here is higher than those other studies, but still clinically relevant in detecting stages of liver disease. Additionally, while many of these biosensors show a shorter response time than the assay demonstrated here, our assay is superior in its sensitivity, width of linear range, and ease of electrode fabrication. While most studies couple ALT to other enzymatic reactions, the second reaction shown here (AKG and H_20_2) is non-enzymatic and therefore fabrication is as simple as mixing Prussian Blue into carbon ink—there is no need for dip-coating, doping or other more complicated fabrication methods. Furthermore, this type of reaction is less temperamental than an enzymatic process, allowing for greater electrode stability and storage at room temperature. By using screen printed electrodes, we have decreased the sample and reagent volumes (up to ~10 fold) needed to perform a complete test (Chang et al., 2007; Jamal et al., 2009; Paraíso et al., 2014; Thuy et al., 2016).

Our assay involves reasonable sample preparation when compared to other studies. At the current stage, our assay involves pipetting of different reagents at different time points, similar to the assay demonstrated by Jamal et al. (2009) and Paraíso et al. (2014). Alternatively, other recent studies pipette mixtures all together, at one time point (Chang et al., 2007; la Cour et al., 2016; Thuy et al., 2016). Therefore, though our assay shows a longer time from start to finish than other assays published in literature, a comparable amount of sample preparation is required when compared to most recent literature.

We have shown that this assay is specific and works over a large range of ALT concentrations. In the future, our assay would benefit from streamlining of sample preparations steps, thereby shortening the time from blood prick to quantification. This assay could also work for AST, by substituting aspartate for alanine, as both enzymes use alpha-ketoglutarate, the molecule exploited here as proxy for ALT concentration.

5. Conclusion

Liver disease represents a major yet neglected public health issue and is particularly burdensome in low-resource settings. The novel, electrochemical assay reported here has the potential to simplify liver function testing, reduce associated costs, and deliver quantitative clinical information. We have demonstrated the ability of our assay to measure levels of ALT quantitatively. We can detect clinically relevant levels of ALT, with high sensitivity and over a wide range of ALT levels. This range encompasses pathology ranging from chronic hepatitis to acute toxic injury. To translate this assay to the POC, testing would need to encompass 1) production of low cost, paper-based electrodes, 2) utilization of a portable electrochemical reader, and 3) validation using both physiologic and pathologic blood samples. Utilizing this assay for prompt quantification of liver enzymes at the bedside, healthcare workers can make appropriate medical decisions to improve patient outcomes.

CRediT authorship contribution statement

Saundria Moed: Conceptualization, Data curation, Methodology, Validation, Formal analysis, Writing - original draft, Writing - review & editing. **Muhammad H. Zaman:** Project administration, Supervision, Funding acquisition, Writing - review & editing.

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Declaration of interests

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2019.02.032.

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