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Flexible Electrochemical Lactate Sensor

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FLEXIBLE ELECTROCHEMICAL LACTATE SENSOR

by

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ABSTRACT

A FLEXIBLE ELECTROCHEMICAL LACTATE SENSOR

Peyton Miesse and Dr. Gymama Slaughter
Old Dominion University, 2020
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Lactic acid is a vital indicator for shock, trauma, stress, and exercise intolerance. It is a key biomarker for increases in stress levels and is the primary metabolically produced acid responsible for tissue acidosis that can lead to muscle fatigue and weakness. During intensive exercise, the muscles go through anerobic metabolism to produce energy. This leads to decreases in the blood flow of nutrients and oxygen to the muscles and increases in lactate production, which in turn cause lactic acidosis. Currently, changes in blood lactate concentrations are monitored by sensors that can be invasive via blood or wearable based sensors that use the enzyme lactate oxidase. Lactate oxidase produces hydrogen peroxide, which is a toxic byproduct and can foul the surface of the sensor. Here, we present the development of a noninvasive wearable electrochemical lactate biosensor for the detection of lactic acid. The bioelectrode was designed with buckypaper (BP), which is composed of a dense network of multi-walled carbon nanotubes. This material was chosen due to its low cost, high conductivity, flexibility, and high active surface area. D-Lactate dehydrogenase (D-LDH) was immobilized on the surface of the BP to facilitate the oxidation of lactic acid. The biosensor was then integrated into a polydimethylsiloxane (PDMS) flexible substrate platform. PDMS was chosen because of its lightweight, flexible, biocompatibility, and conformal properties. The sensor is designed to be placed on skin in order to measure the concentration of lactate in sweat. The concentration of lactate in sweat has been shown to be a

good biomarker for evaluating the severity of peripheral occlusive arterial diseases and damage in soft tissue. The lactate biosensor developed in this work exhibited a dynamic linear range of 5 mM to 45 mM lactic acid with a good sensitivity of 1.388μA/mMcm^2. It can measure higher than the average lactate concentration in sweat during exercise, which is 31mM. This electrochemical biosensor has the potential to be used for the real-time detection of lactic acid concentration in sweat, suggesting promising applications in clinical, biological and sports medicine fields.

This thesis is dedicated to my parents Jeanne and Greg, brother Tyler, fiancé Nick Kovach, Costa, and Oakley

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CHAPTER 1

INTRODUCTION

Background

Wearable sensors are becoming widely popular throughout the medical field due to the benefits that are derived from the sensors being noninvasive as well as their capabilities to monitor ongoing physiological problems arising in consumers. Patients with wearable sensors can receive personalized health assessments and examinations [1]. Flexible sensors can play a key role in early detection of certain diseases and injuries because they are typically cost effective, lightweight, and easy to fabricate [2]. Wearable technology that is currently on the market for consumers has wrist-worn, textile, or strap mounted formats that require bulky power sources. With current technological advances, electrochemical sensors have increasingly become smaller in size while maintaining their abilities to be used as analytical devices [3]. These devices have become more user friendly, inexpensive, comfortable, smaller in size and easier to operate [4]. Typically, this technology is limited to high-level physiological signals such as temperature, heart rate, and skin conductance [3]. Recent research has shown an increase in demand for wearable sensors, such as epidermal sensors, which allow consumers to easily monitor their health throughout the day [5]. Many sweat sensing devices are designed to be worn in the form of a patch, watch, or temporary tattoo that can measure analytes such as glucose, lactate, ammonia, ethanol, pH, and hydration biomarkers [3]. Using personalized healthcare devices can enable a person to prevent illnesses from getting worse or be coming incurable.

One of the most widely developed sensors is the electrochemical sensor. Originally, electrochemical methods were traditionally used for in vitro diagnostics systems to measure pH, gases, and electrolytes in blood [6]. The three major electrochemical methods are potentiometry, amperometry, and conductometry (Table 1). This is discussed in more details in Chapter 1. Briefly, potentiometry is based on the electrical potential difference between two electrodes when the current is zero. Amperometry is when a constant potential applied between two electrodes shows the oxidation or reduction of an electroactive species. Finally, conductimetry relies on the applied potential between two inert metal electrodes. This is used to determine if the analyte changed the conductivity of the sample. The electrochemical sensor developed by this project utilizes amperometry, which uses electrocatalytic activity to detect a particular biological analyte [7].

Table 1. Different electrochemical methods

Electrochemical Methods	**Summary**	**Advantages**	**Limitations**
Potentiometry	Use a zero-current potentiometry which current is of controlled amplitude is applied to the working electrode	• Rapid response • Reproducible • Simple technique	• Unable to detect lower limit ions
Amperometry	Monitor the current associate with the oxidation or reduction of an electroactive species that is used in the recognition process	• High selectivity and sensitivity • Reproducible • Inexpensive	• Introduce interferences as a result of oxidation of other matrix components
Conductometry	Two electrode device to measure the conductivity of the electrolyte adjacent to the electrode surface	• Does not require reference electrode • Inexpensive • Miniaturization	• Sensitivity decreases in the presences of non-reacting ions in the solution • Low specificity

A common way to analyze and detect lactic acid levels is through blood collection. This method is less efficient than using a non-invasive sensor to measure sweat. Invasive sensors that rely on blood are difficult to use during exercise and may not provide lactate levels in real time while exercising. Human sweat lactate concentration has been reported to show a positive correlation to results that have been derived from lactate concentrations in blood [8]. Venous blood lactate concentrations before exercise are between 2.5 - 3.5 mM and sweat lactate concentrations are between 13.7 - 27.1 mM [9]. This indicates that perspiration contains a higher concentration of lactate before and after exercise than blood. Sweat also allows for a less invasive way of sampling without the risk of infection.

Lactate is an important metabolite of the anerobic glycolysis pathway and serves as a biomarker for lactic acidosis. During anerobic conditions, pyruvate is converted to lactate by the enzyme Lactate dehydrogenase (LDH). Lactic acid is produced at physiologic pH ranges [10]. Lactic acid exists as L-(+) lactate, which is a normal intermediate in the mammalian metabolism. The D-(-) is produced by microorganisms, algae, and plants. When assessing a patient for elevated lactic acid levels during surgery or critical care, L-L-lactate concentration in the blood is measured. Elevated lactate levels in the blood can indicate ischemic conditions such as heart failure, shock, and respiratory insufficiency [11]. Additionally, lactate levels can be altered due to diabetes or absorptive abnormalities of short-chain fatty acids in the colon. When there is a shortage of oxygen to the muscles, an increase in lactate production occurs. An increase in lactate levels can be a key indicator for tissue acidosis leading to muscle fatigue and weakness [12]. Recently, lactate has been found to be the major cause of acidification in the microenvironment of cancer cells, which in turns helps with cancer diagnosis [13].

There are different ways to measure lactate, such as through blood, sweat, saliva, and tears. Blood has been the primarily biofluid for measuring lactate. Drawn blood must be analyzed immediately, as concentrations can increase by 70% in 30 minutes at room temperature due to glycolysis. The sampling bio-matrix can affect the biosensor response to the analyte, causing potentially skewed results. Some sensors are implantable, which allows for continuous monitoring of lactate through blood but is extremely invasive. This can lead to a high risk of infection [14]. Using a noninvasive technique to measure lactate concentrations decreases the risk of infection. Saliva can be used to measure lactate during anerobic metabolism. Saliva has been shown to have a high correlation with blood lactate and human saliva lactate level is about 0.2 mM. This allows for saliva to be used as a key diagnostic tool in measuring lactate. Tears can also be used to measure lactate level. The concentration is around 2 - 5 mM in the tear fluid [14]. Since these techniques are non-invasive, this allows for simple, safe, and stress-free procedures. Overall, measuring lactate through sweat has been shown to have higher concentration of lactate than using blood, saliva, or tears to detect lactate. Sweat has been demonstrated to be an easily accessible non-invasive bodily fluid that can provide diagnostic information in a quick and efficient manner [15].

We have developed a noninvasive enzymatic flexible biosensor that monitors lactate levels with a high sensitivity of 1.388 $\mu A/mMcm^2$. Different enzymes have been used as biorecognition elements in electrochemical sensors to detect lactate. D-LDH was selected for the development of this lactate biosensor to oxidize lactate, since it does not produce the toxic hydrogen peroxide byproduct observed with lactate oxidase (LOD). Hydrogen peroxide has been shown to affect the performance of the electrode [16]. The material that was selected for the working electrode was buckypaper (BP), which is composed of a dense network of multi-walled carbon nanotubes. The benefits of using BP are that it is easy to reproduce, can allow for large-scale production, and has

controllable porosity. The BP also allows for enhanced electrical conductivity, which increases charge transport [17]. The biosensor is then placed on polydimethylsiloxane (PDMS), which is a silicon-based elastomer [18]. PDMS is flexible and elastic, which makes it a good base for the sensor (shown in Figure 1).

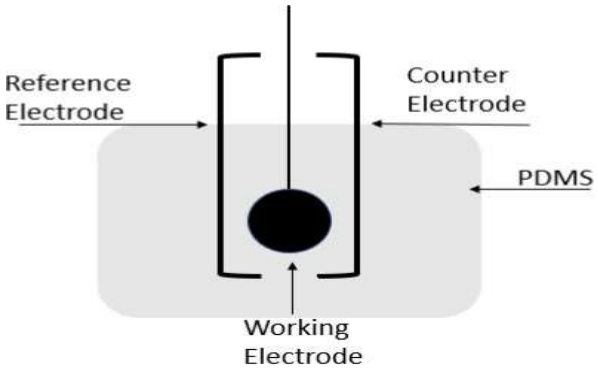

Figure 1. Schematic illustration of the flexible wearable electrochemical lactate biosensor.

Thesis Statement

This thesis focuses on the development of a flexible lactate sensor for the medical and sports medicine fields to detect lactate. The biorecognition element, D-LDH, which has high catalytic activity in the presence of lactic acid, was immobilized on BP to maintain a stable environment for lactic acid biosensing.

Author Contribution

I developed a flexible lactate sensor after a year of research and work. I came up with a protocol that can be used to replicate the sensor fabrication process. The design of the working

electrode was made into a circle with a diameter of 9 mm. I made the biosensor with and without enzymes and chose to move forward with an enzyme-based biosensor because it performed well at physiological conditions. The tests that were conducted were a cyclic voltammetry, chronoamperometry, pH, and temperature profiling to study the characteristics of the operational biosensor.

Thesis Outline

The purpose of Chapter 1 is to provide background about the thesis and introduce the reader to biosensors and lactate in the body.

Chapter 2 focuses on different biosensors that are used for lactate sensors. This will provide details on non-enzymatic sensors and enzymatic sensors.

Chapter 3 focuses on lactate in the body. This will provide details on the biochemistry of lactate and the different ways to sense lactate through sweat and blood.

Chapter 4 focuses on the discussion of the enzymatic lactate biosensor system. The fabrication methods and the materials used to prepare the lactate electrode will be discussed. All the experimental data and results are presented in detail.

Chapter 5 briefly describes the studies performed and future outlook of the lactate biosensor.

CHAPTER 2

BIOSENSORS

Biosensors

The history of biosensors began with Leland C. Clark who invented a device that was able to measure oxygen in blood, water and other liquids. This led Leland to become the "Father of Biosensors." In 1962, Leland C. Clark began the development of an enzyme electrode [19]. Over time, different research fields have made advancements to biosensors which have led to more sophisticated and reliable sensors. A biosensor is a self-contained integrated device that is able to quantify analytical information using a biological recognition element, which is in direct spatial contact with a transduction element [20]. Biosensors can be used for multiple applications and are geared towards improving quality of life [21]. Biosensors are utilized in a wide range of areas such as defense, homeland security, agriculture, food and safety medicine, and pharmaceuticals [22]. Biosensors can be classified into four types: electrochemical, optical, piezoelectric and thermal sensors. Electrochemical sensing requires three electrodes which are the working electrode, counter electrode, and the reference electrode. Electrochemical sensors have a high sensitivity and fast response time. In comparison, optical sensors rely on change in the refractive index, absorbance, and fluorescence properties of the specific analyte. The downfall of this type of sensor is that it is difficult to miniaturize and is much more costly that an electrochemical sensor.

Piezoelectric sensors are used to measure the sensitivity of mass to frequency. Theses sensors are sensitive to change in mass, density, or viscosity of the sample in contact with its active surface [23]. Finally, thermal sensors measure the thermal energy that is absorbed or released in

the biochemical reactions [24]. This application has many drawbacks such as poor sensitivity and non-specific heating effects, which have resulted in its poor reputation [25]. Since electrochemical sensors have many advantages (e.g., the simplicity of the design of the system, low cost and simple instrumentation), the focus of the rest of this chapter will be on electrochemical biosensors, wearable biosensors, nonenzymatic biosensors, and enzymatic biosensors.

Electrochemical sensors

Electrochemical sensors must contain two basic functional units, which are a receptor (biorecognition element) and a physico-chemical transducer. The receptor is made of a biological component (such as enzyme, antibody, or DNA) while the receptor modifies the analyte concentration into a physical or chemical signal with a distinct sensitivity. The receptor's purpose is to be highly selective towards the chosen analyte and free from other interfering chemical species. The transducer will then convert the signal generated by the receptor and analyte interaction (as shown in Figure 2) [26].

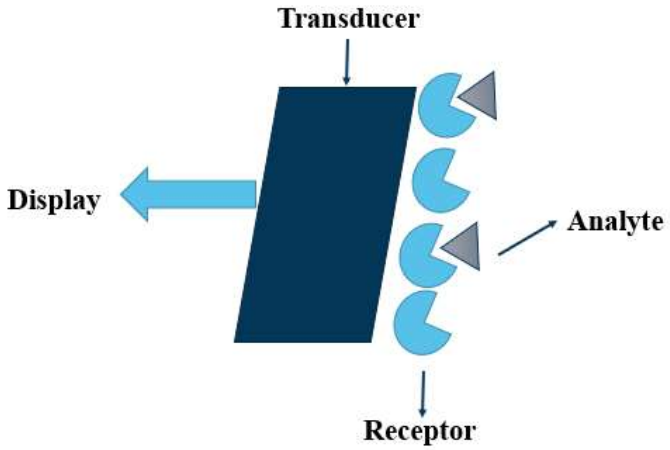

Figure 2. Schematic of the important components of an electrochemical sensor. Adapted from A. J. Bandodkar et.al [26]

The reaction of electrochemical biosensors occurs when the potential is applied to the working electrode and the resulting current is measured against time. The reduction and oxidation of a redox couple are associated to the potential (E) by the Nernst equation:

$$E = E_0 + \frac{RT}{nF} ln \frac{C_{oxi}}{C_{red}} \quad (1)$$

In this equation, E_0 is the half-cell potential, F is the Faraday constant, T is the temperature, and C_{oxi} and C_{red} are concentrations of reduction and oxidation species. Overall, the resulting electrical signal is related to the recognition process of the specific analyte and proportional to the analyte concentration [23].

In this regard, Hernández-Ibáñez et al., [27] designed a screen printed electrochemical biosensor that can detect lactate within an embryonic cell culture. The electrochemical lactate biosensor used multiwalled carbon nanotubes for the working electrode. Carbon materials were utilized for electrochemical sensing *in vitro*. The carbon material has many advantages when used as an electrochemical sensor. This material has high conductivity, large surface area, surface can be easily modified, fouling resistance, high reproducibility and has high electrocatalytic activity. The majority of biosensors use electrochemical detection for the transducer due to the low cost, portability and simplicity of construction [28]. For example, Liu et al., [29] recently reported that electrochemical sensors are powerful analytical tools which possess the capacity for rapid detection of biomarkers in clinical species. This allows for the sensors to be small and have a high sensitivity.

Electrochemical sensors contain a biological recognition element, which can be enzymes, proteins, antibodies, nucleic acids, tissues or receptors that react with the targeted analyte and produce an electrical signal [28]. Electrochemical sensors can detect many biological markers in bodily fluids such as sweat, saliva, urine, and blood [30]. The advantage of electrochemical sensor over other types of sensors is it is able to sense the materials, which are present within the host without damaging to the host system [31].There are three types of electrochemical sensors: potentiometric, amperometric and conductometric.

Potentiometric sensors are a chemical sensor that measures the potential difference between two electrodes under the conditions of no current flow [32].These sensors have been used since the 1930's and are still being used due to their simplicity and low cost. These are used for determining inorganic or organic ions in medical, environmental, and industrial analysis [33]. The most common potentiometric device is a pH electrode that has been used for several decades. Potentiometric methods use a zero-current potentiometry in which the current is of a controlled amplitude and is applied to the working electrode [34]. The most used potentiometric sensor is an Ion-Selective electrode (ISEs). The advantages of these sensors are that they have a rapid response, good reproducibility, and simple measuring techniques. Classical ISEs are symmetrical, which means there are two solutions. First, is the test solution and then the inner solution with constant concentration of ionic species. The sensor typically uses an Ag/AgCl reference electrode that is in contact with the internal solution that contains chloride ions at constant concentrations. The response of the potentiometric sensors mostly depends on the bulk properties of an ion-selective membrane/film. [35]. The limitations of potentiometric wearable sensors is that the instrumentation is large and it is unable to detect lower limits of ions [36] .

Both potentiometric and amperometric transducers are commonly used for electrochemical sensors. However, amperometric sensors monitor the current associated with the oxidation or reduction of an electroactive species that is used in the recognition process [37]. The sensor generally has a short response time which makes it a useful application for medicine, food technology, and the environmental industry [38]. The design of an amperometric sensor is composed of two or three electrodes: a working electrode that is immobilized with an enzyme, a counter electrode, and a reference electrode (as shown in Figure 3) [39]. Amperometric sensors are known to be inexpensive, provides reproducible results, and highly sensitive. The sensor is a chemically modified electrode that is very selective and sensitive [40]. There has been an increase in the last 10 years for enzyme-based amperometric biosensors due to the high selectivity and sensitivity of the amperometric signal. The limitation of amperometric sensing is that not all protein analytes are capable to serve as redox partners in electrochemical reaction. These devices are mostly used in electrochemistry for the electrochemical reaction of the analyte at the working electrode [41].

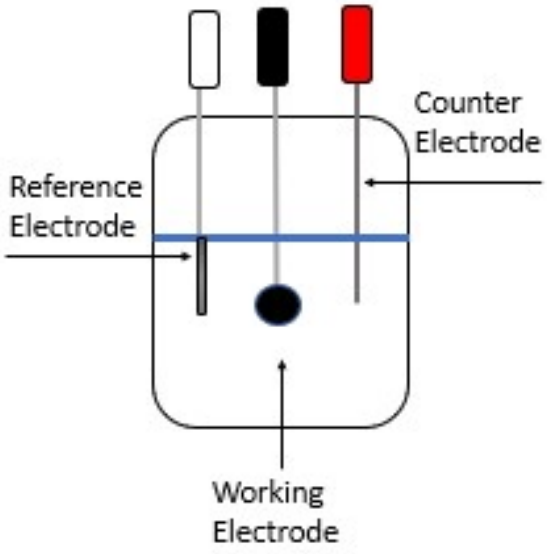

Figure 3. Schematic of an amperometric sensor in solution with a working, reference, counter electrode.

Another electrochemical sensor is conductometric. This sensor is involved with the measurement of conductivity at a series of frequencies [42]. This method must have a conductive liquid, which results from the dissociation of the dissolved substance of an electrolyte into ions and the generation of an electrical field. Once there is a potential difference applied to the electrode then ion movement will occur. Negative charge moves towards anodes that have a positive charge while positive charge moves towards the cathode. The ion movement towards the electrodes causes current in the electrolyte (as shown in Figure 4) [43]. The material of the sensor is conductive to the analyte that is present. The advantages of these sensors are that there is no reference electrode required and the sensor can be low cost [44]. A limitation of conductometric techniques is there must be a reaction ion in the solution, or the sensitivity will decrease. Another disadvantage is that

it has a low specificity and cannot distinguish between reactions that cause an artifact [43]. Both limitations will cause an error for this method when testing.

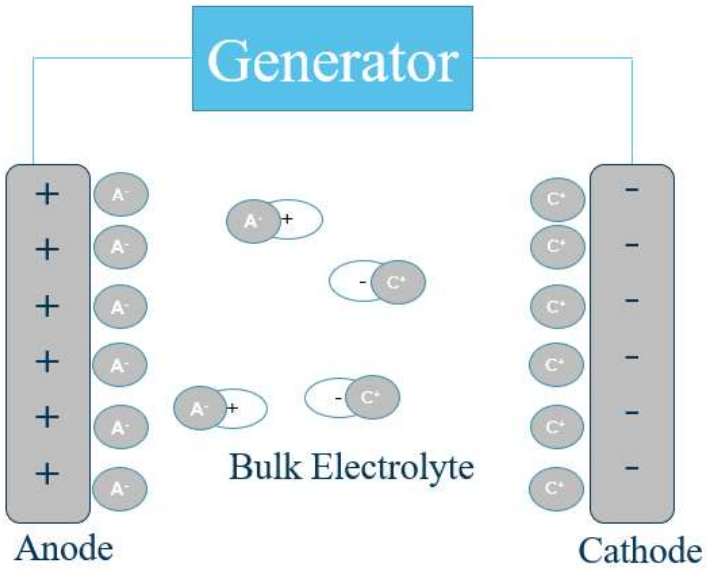

Figure 4. Ion migration in the solution and electrolyte conductivity. Adapted from N. Jaffrezic-Renault et. al [43]

Overall, electrochemical sensors are very common biosensors due to their wide range of potential applications. These sensors are usually inexpensive and simple to construct. Further research and investigation are still ongoing in this field to make the biosensors miniaturized, cheaper, more accurate, and provide faster feedback in real time.

Wearable sensors

Wearable technology has become a huge market for the medical field due to its convenience and real time monitoring. The wearable technology market is expected to be close to $70 billion in 2025 [45]. Some wearable sensors are in the form of a panic buttons for emergency help, and this sensor has shown to be commercially successful [46]. The objective of wearable sensors is to continuously monitor wearer's physiological measurements such as heart rate and skin temperature. There was a significant increase in wearable devices in 2015; and now, there are more than 500 different health related wearable devices [47]. Since there has been an increase in monitoring human performance, there has been a significant amount of research on wearable sensors. Wearable sensors do use noninvasive chemical analysis of biofluids such as sweat, tears, saliva, and interstitial fluid. These biofluids can be readily accessed without disrupting the outermost protective layers of the body's skin and without using blood [48].

Tear fluid can be used to monitor glucose as well as intraocular pressure from the human eye. Intraocular pressure can be a risk factor of glaucoma, which is a leading cause of human blindness [49]. A contact lens is a wearable sensor that collect tear fluid. It can allow for continuous monitoring of physiological conditions. The contact lens uses soft material that offer flexibility and minimize eye irritation for the user [48]. Kim et al., [49] designed a multifunctional contact lens that can detect glucose within the tears as well as the intraocular pressure. A limitation of contact lens biosensor is that it can be affected by repeated eye blinking due to the soft material, and the contact lens can lead to obstructed vision. Another noninvasive wearable sensor that uses a body fluid is saliva. Saliva-based sensors can detect various biomarkers to improve clinical diagnosis and treatment. They are convenient, safe, and cost effective for monitoring diseases such as diabetes and renal disease [50]. Mannoor et al., [51] designed a graphene nanosensor that

was printed onto water-soluble silk. This biosensor was then placed on the tooth enamel or tissue which monitored pathogenic bacteria. The limitation with using saliva for a wearable device is that brushing teeth, processing food, and smoking can affect the composition of saliva or damage the sensor [52].

Sweat-wearable sensors have only been around for about a decade, and many recent advancements in wearable sensors have been studied intensively [53]. A common sensor that is used to measure human performance is a wearable lactate sensor. The sensor is able to sense lactate through perspiration of the skin [40]. These sensors are non-invasive and are very convenient to the user. A tattoo based paper biosensor has been examined on the epidermal surface on the human neck and under various forms of mechanical strain [5]. However, this sensor needs to be examined on other locations of the body and under different environments to determine its full potential and limitations. Two types of material that are used for non-invasive electrochemical sensor for monitoring sweat are fabric/flexible plastic-based devices and epidermal based sensors. Epidermal sensors can be tattoo-based paper which allows for direct and continuous contact with the skin. Jia et al., designed a flexible printer temporary tattoo biosensor that can noninvasively monitor lactate levels. There are disadvantages to tattoo sensors. The tattoo sensors are one time use only and cannot withstand stretching stress [54]. The fabric/flexible plastic based biosensor material tends to be electrochemical sensors that are screen printed on these materials that have large surface area [26]. These types of sensors tend to be low cost. An easily reproducible substrate such as polydimethylsiloxane (PDMS) is used because of its moldable and flexible properties. PDMS is comfortable to wear on the skin and it can cover the sweat collection area and prevent the sweat from evaporating [55]. PDMS has been used due to its high transparency and superior mechanical flexibility [56]. It has been used mostly for microchips [57], micro pumps [58], and electronics on

the skin [59]. PDMS is the most widely used flexible substrate material due to its excellent comprehensive performance [60]. Kim et al, designed a flexible triboelectric nanogenerator using PDMS and CNT nanostructures [61]. PDMS was used due to its high flexibility and stretch ability, since the sensor will be placed on a human body during human activity. Wu et al., [62] fabricated a three electrode sensor on PDMS to detect hydrogen peroxide. The use of PDMS allowed for miniaturization, reproducibility, light weight and flexible microstructures of the sensor.

Non-enzymatic sensors

Non-enzymatic sensors' ability to detect an analyte is based on electrocatalytic reaction of non-biological material such as metals, alloys, and metal oxides, like copper oxide and nickel oxide [63]. These sensors will have a longer lifetime than enzymatic sensors because they do not contain any biological components on the surface of the sensor [64]. The non-enzymatic sensor is less expensive to produce since the sensor does not require a biological component such as costly enzyme [65]. Recently, Jiang et al., designed a glucose sensor by coating copper on carbon nanotube rubber fiber [66]. This allowed the sensors to be highly stretchable and able to withstand large strains of up to 60% with negligible influence on its performance. During the detection of glucose, the molecule binds to the electrode and then its electrochemical environment changes. This biorecognition event indicates that the electrochemical catalyst can oxidize the glucose molecule. Enzyme glucose sensors have shown to be effective under various pH, temperature, and humidity environments. This type of sensor has proven to have a higher stability than the enzymatic sensors. Enzymes sensors have been shown to lose almost half their sensitivity within the first ten days [67], whereas these sensors have been proven to be more reliable.

Non-enzymatic lactate sensors have been developed to use nickel oxide to detect lactate. Nickel oxide can easily be fabricated with conventional solution-based methods. Zaryanov et al.,

designed a screen printed non-enzymatic lactate sensor. The working electrode surface was modified using electropolymerization of 3-aminophenylbornoic acid with imprinting of lactate [67]. The lactate sensor had a lactate detection range from 3 mM to 100 mM in the solution 0.1M KCL. This sensor was able maintain its sensitivity through 6 months of storage at room temperature. The response time for this sensor was reported to be between 2 - 3 minutes. The limitation of non-enzymatic lactate sensors is it exhibits relatively low sensitivity compared to enzymatic sensors and it has a slow response time of ≥2 minutes, versus rapid responses in enzymatic sensors [65,70].

Enzymatic Sensors

Enzymes are proteins that are biological catalysts for biochemical reactions. Enzymes are usually used as bioreceptor molecules for biosensing [69]. When a biosensor uses enzymes, it will be combined with a transducer in which it will produce a signal proportional to target the analyte concentration. This signal occurs from a change in proton concentration, release or uptake of gases, light emission, or absorption. The transducer will then convert the signal into current, potential, thermal or optical means [70]. Enzymes can recognize a specific chemical reaction. Enzymes are categorized in six classes which include: dehydrogenase, oxidases, peroxidases, and oxygenase. These enzymes are involved in an oxidation/ reduction reaction of the substrate via transfer of hydrogen or electrons [69] . Enzyme biosensors are used for clinical analysis, food safety control, or disease monitoring purposes. This is due to the biosensor having a high sensitivity and specificity, portability, cost effectiveness, and possibilities for miniaturizations [71].

The disadvantages of using enzymes are that they have a low stability and a lack of long-term operational stability. To overcome these issues and make enzyme utilization more desirable, immobilization of enzymes have become a focus. Immobilization of enzymes is when the enzymes

are physically or chemically confined or localized in a region of space of their catalytic activities [72] . Immobilized enzymes are more resistant to environmental changes and are more stabilized [73]. There are different immobilization techniques used to improve the stability, sensitivity, response time, reproducibility, and selectivity [74]. The most common immobilization strategies are covalent binding, entrapment or encapsulation, cross-linking, and adsorption.

Covalent binding method has a strong binding which allows for the immobilized enzyme to be stable (shown in Figure 5). This method occurs when stable complexes between functional groups on enzyme molecules and a support matrix are formed through covalent bonding [75]. Covalent binding is associated with enzymes that support owing to their side chain amino acids like arginine, aspartic acid, histidine, and degree of reactivity based on different functional groups [76]. The covalent binding will go through two stages: the activation of the surface using linker molecules such as glutaraldehyde and the enzyme covalent coupling to the activated support. The glutaraldehyde, which is the multifunctional reagent, will act as the bridge between surface and enzyme by covalent bonding [75] . Fu et al., modified the enzymes on the surface using covalent binding which enhanced enzyme activity and stability [77]. The concept was conducted by covalently binding the peptides to β-galactosidase through enzyme immobilization. This exhibited higher specific activity and stability in the peptide-modified surface. Overall, covalent binding provides for strong bindings between enzymes and support matrix. This leads to less leakage of enzymes from the support surface. However, covalent binding has a high risk of enzyme denaturization when the enzymes undergo the chemical modification process. This immobilization process increases enzyme stability but decreases the enzyme activity in affinity reaction and is poorly reproducible [71].

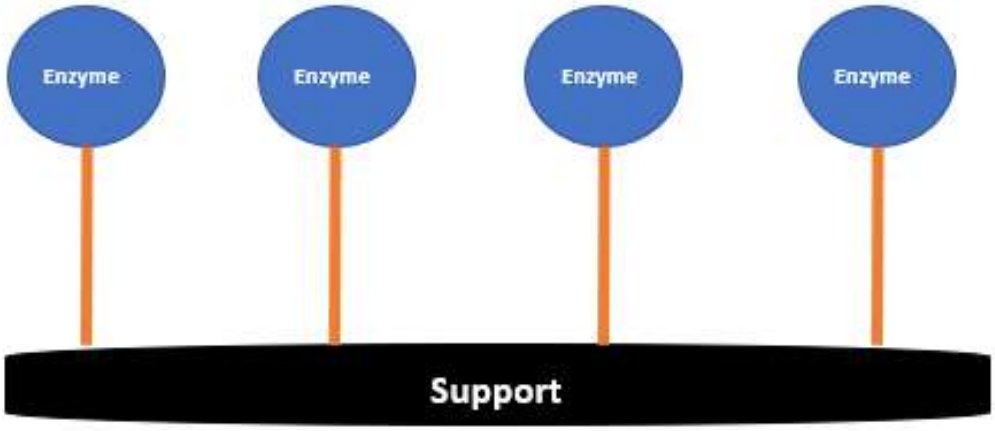

Figure 5. Schematic shows the enzyme immobilization covalent binding

Entrapment is another method for enzyme immobilization. The enzymes in this method are not attached to the support surface but are entrapped in a gel matrix that has minimal leaching of enzymes and high stability of the enzyme. The enzyme is confined within a polymer lattice network, the enzyme does not chemically interact with the entrapping polymer [75]. The enzyme immobilization methods, entrapment and encapsulated are shown in Figure 6. These methods can be used in the chemistry field, biomedicine, biosensors, and biofuel due to the use of nanofiber and pristine material to perform the entrapment [76]. Ivnitski et al., [78], designed a glucose oxidase sensor on carbon nanotubes, where the glucose oxidase was immobilized on the surface of the carbon nanotubes. A silica matrix was applied to the electrode surface to entrap the immobilized enzymes. This helped stabilize the enzyme activity and support electrical conductivity. However, the gel matrix can interfere with the immobilization of the enzymes,

limiting the loading capacity of the enzyme. This method also leads to enzyme leakage, due to the pore size of the support matrix being too large. To overcome this issue the membrane porosity must be adjusted according to the different molecule sizes of the enzyme to prevent leakage [71].

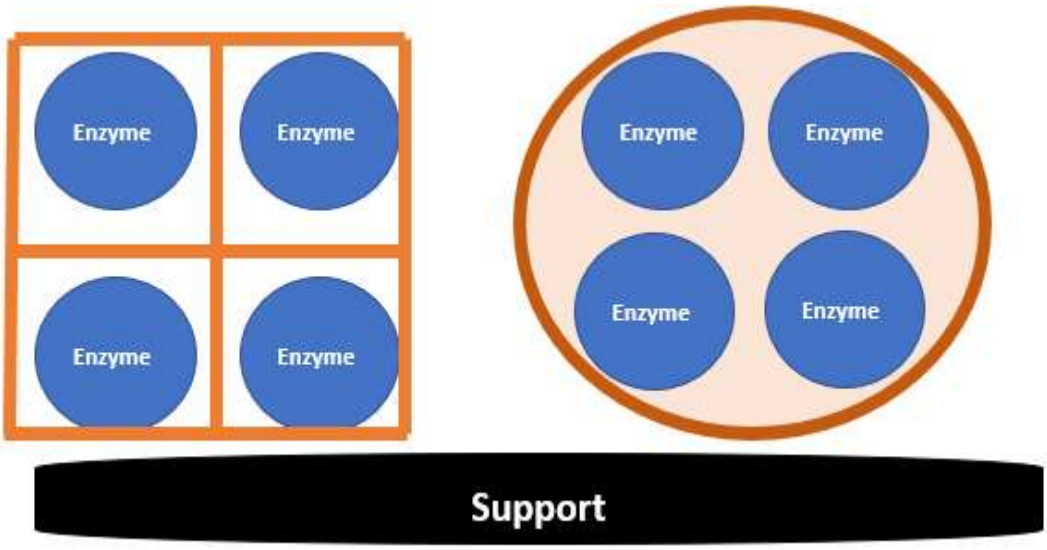

Figure 6. Schematic shows the enzyme immobilization entrapment and encapsulation.

A third method is cross-linking, which allows for a highly strong and stable bonding between enzymes and the support substrate (shown in Figure 7). This method is performed by the formation of intermolecular cross-linkages between the enzyme molecules by covalent bonds, which is carried out with the assistance of a multifunctional reagent. This reagent will act as linkers to connect the enzyme molecules into three-dimensional cross-linked aggregates. Cross-linking reagents are glutaraldehyde, bisdiazobenzidine, and hexamethylene diisocyanate that are typically used to cross-link enzyme molecules by the reactions of the free amino groups of lysine residues on the reactive site of the neighboring molecules [75]. During the immobilization method, the

enzyme aggregates produced by precipitation of enzymes from aqueous solution by addition of organic solvents. This can enhance longevity and operational stability of the electrode [76]. This immobilization method has shown to have a strong chemical binding of enzyme biomolecules with minimal enzyme leakage. Nonetheless, glutaraldehyde can result in severe enzyme modifications and lead to enzyme conformational changes and loss of activity [71].

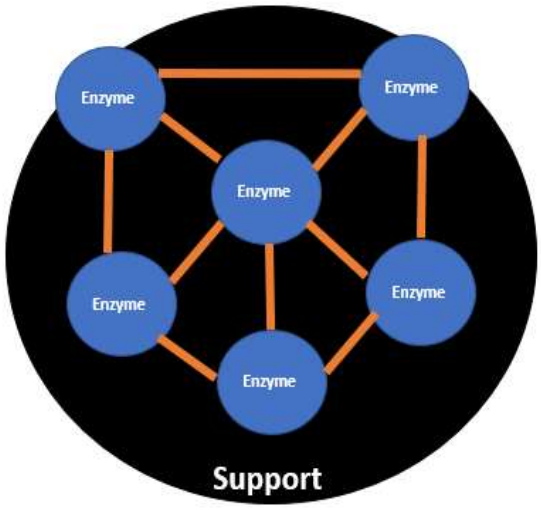

Figure 7. Schematic shows the enzyme immobilization cross linking.

Finally, the easiest method is adsorption (shown in Figure 8). The adsorption method occurs when the enzymes are absorbed onto the supporting matrix through weak non-specific forces, such as Van der Waal's forces, electrostatic and hydrophobic interactions [75]. This method is performed by depositing the enzymes on the electrode surface or by dipping the electrode in the enzyme solution for a set duration. This sustains the enzyme activity. This method is simple, inexpensive, and is less destructive to the enzyme activity than the other methods. The binding is

weak, thereby preventing the active sites of the enzyme from disturbing and allows the enzyme to retain its activity [79]. Kibarer et al., [80], fabricated the surface of a petroleum-based activated charcoal by the adsorption of urease. This allowed for stability of the enzyme and enhanced the activity. The limitation of this method is that the enzymes can be subject to many changes that could weaken the biosensor signal [71]. However, to avoid this, Nafion is used as a protective coating on the surface of the enzyme immobilized electrode in order to prevent enzyme leaching, as well as to selectively screen against interfering analytes [81]

Figure 8. Schematic shows the enzyme immobilization process adsorption.

Two enzymes that have been frequently used during the design of lactate sensor are lactate oxidase (L-LOD) and lactate dehydrogenase (D-LDH) [82]. This is due to their simple enzymatic reaction and simple sensor design fabrication. Currano et al., designed a wearable lactate sensor using the enzyme lactate oxidase [3]. The enzymatic reaction of lactate with lactate oxidase produces hydrogen peroxide. The rate of hydrogen peroxide produced is dependent on the rate of the lactate/lactate oxidase reaction. The limitation to using lactate oxidase is it requires a high oxidation potential, which leads to interferences caused by electro-oxidizable species [82]. L-

LOD can be obtained by a bacterial source such as Pediococus, Aerococcus viridans, and Mycobaterium smegmatis. In the presence of dissolved oxygen, L-LOD catalyzes the oxidation of L-Lactate to pyruvate and will form hydrogen peroxide, which is electrochemically active, and will either be oxidized or reduced to give a current proportional to L-Lactate concentration. Due to the reduction of oxygen, oxidation of hydrogen peroxide is required for detection [82]. The electrochemical reaction involved lactate biosensor is summarized as follows:

$$L - lactate + 0_2 \xrightarrow{L\text{-}lactateoxidase} Pyruvate + H_2O_2 \quad (2)$$

$$H_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

In Eqn. 2, the electrons are transferred through the FAD cofactor in the enzyme structure. Oxygen is a main component in the enzymatic reaction which has motivated researchers to design lactate sensors based of the depletion of oxygen. These sensors have less interference because the sensor functions at a cathodic potential. The signal depends on the ambient oxygen contraction and must be assumed that oxygen is constant, or it must be monitored. Due to monitoring the oxygen increase, the complexity of the sensor design leads to extra cost [68]. To overcome this issue of monitoring the oxygen concentration, D-lactate dehydrogenase (D-LDH) is used. This enzyme works in the presence of a co-enzyme NADH or NADP. The electrochemical reaction is as follows:

$$L\text{-}lactate + NAD \xrightarrow{L\text{-}lactate\ dehydrogenase} pyruvate + NADH \quad (4)$$

The D-LDH catalyzes the oxidation of lactate to pyruvate in the presence of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) [83]. The reduced NADH can be amperometrically detected. Baingane et al., designed a self-powered electrochemical lactate biosensor that was immobilized with D-LDH [84]. The sensor showed that D-LDH has a high catalytic activity when it reacts with lactic acid. However, difficulty incorporating the coenzymes NAD^+ /NADH into the biosensor and most often the high oxidation poses problems as additional parameters must be improved. This is a reason that L-lactate oxidase has gained more attention in the development of the lactate sensor [68].

CHAPTER 3

LACTIC ACID

Lactic Acid

Lactic acid is an organic compound that is found throughout the human body (shown in Figure 9). The accumulation of lactate has been associated with impaired sports performance due to lactic acidosis. This research began in the 1920's by British physiologist A.V. Hill. He hypothesized that a decrease in pH derepress the cell excitability and will force a muscular contractile force. However, over the years, advances in technology have shown that lactate is a valuable energy substrate for various physiological systems which include the brain, heart and skeletal muscle [85].

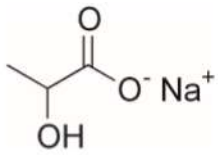

Figure 9. Lactic acid molecular structure

Lactic acid is mainly associated with anaerobic metabolism, which is the creation of energy through the combustion of carbohydrates in the absence of oxygen. During muscle activity, glucose is converted into pyruvate through glycolysis, which produces Adenosine triphosphate (ATP) in the process. With enough oxygen supply, ATP is produced continuously from pyruvate

in the Krebs cycle. Once there is a depletion of oxygen during physical exertion, the anaerobic process is initiated. Pyruvate will then be converted into lactate by the enzyme lactate dehydrogenase which is found in muscles [10]. The lactate metabolism in muscles is shown in Figure 10, where the liver metabolizes lactate. Pyruvate undergoes the metabolic pathway gluconeogenesis to convert pyruvate into glucose. Then glucose goes back into the tissues as an energy source. Under normal conditions the lactate is cleared by the liver with small additional clearances by the kidneys [86]. The liver accounts for 70% of the lactate clearance [87]. If the liver doesn't metabolize the lactate, it can lead to lactic acidosis.

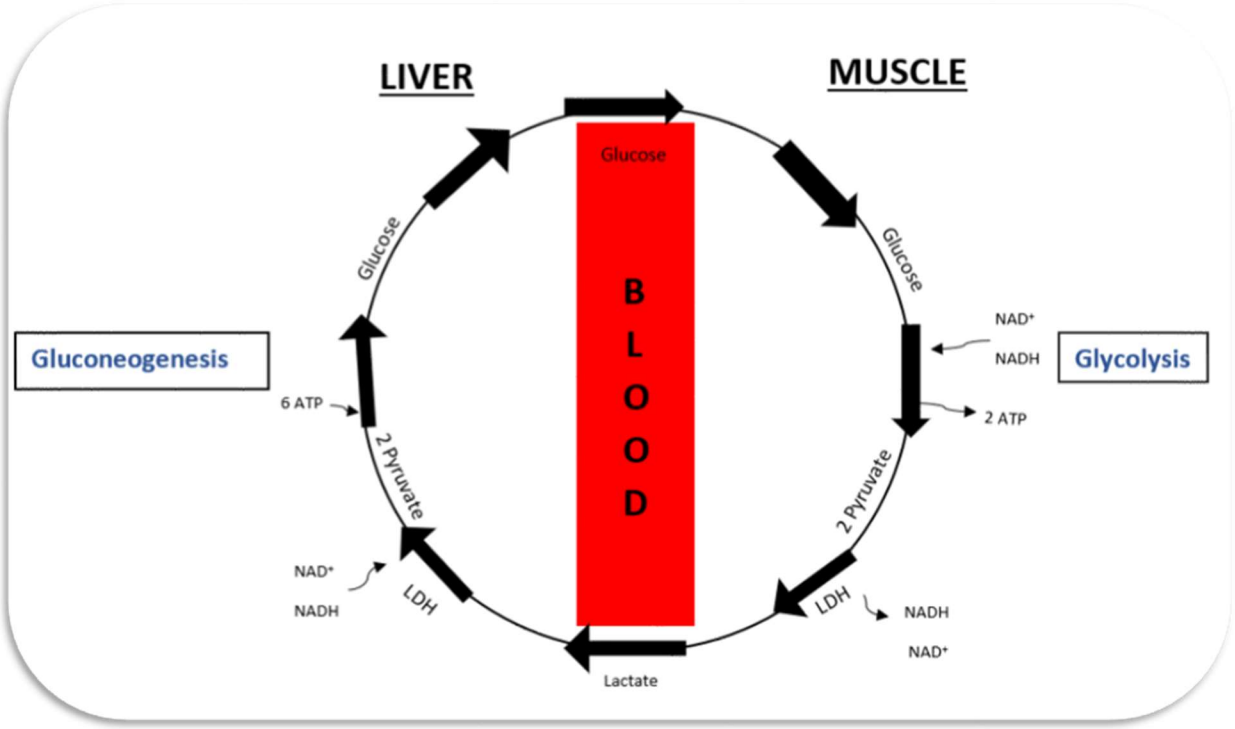

Figure 10. Outline of the Cori cycle. The depletion of oxygen supply, pyruvate will be converted into lactate, thereby assuring regeneration of NAD^+ from NADH. The glycolysis process will lead to the production of ATP.

Lactic acidosis occurs when the body is unable to metabolize the increased amount of lactate in the muscles. This can lead to muscle fatigue and weakness. This is also associated with an increase in mortality in patients with shock and sepsis [88]. There are two classifications of the causes of lactic acidosis [62]. Type A is caused when there is a deficiency in oxygen in the tissue, which increases lactate levels. This can also be developed by shock, such as cardiac failure which has a mortality greater than 80% [90]. Sepsis can also be a cause of Type A lactic acidosis, and this is due to the lack of oxygen during the development of the anaerobic glycolysis within tissues [91]. Type B is from toxin induced impairment of cellular metabolism. There are three categories for Type B. Type B 1 occurs with different diseases such as diabetes, liver disease, renal insufficiency and infection. Type B 2 is linked with drugs and toxins. The last type is Type B 3, which includes congenital errors of the metabolism [90]. Metformin is a commonly prescribed medication for type 2 diabetes. People that typically are affected by metformin-associated lactic acidosis often develop renal impairment from dehydration. This reduces the clearance of the medication resulting in increasing levels of metformin levels in the plasma [92].

Table 2. Different Types of Lactic Acidosis

Lactic Acidosis	Causes	Examples
Type A	• Impairment of tissue oxygenation • Hypoperfusion • Hypoxia	• Septic • Regional ischemia • Seizures • Shock
Type B	• Toxin induced impairment of cellular metabolism	• Medication (metformin) • Diabetic • Liver disease • HIV • Ethanol intoxication

Lactic acid is essential for diagnosing patients' conditions during intensive care as well as during surgery. If a patient has elevated lactate levels, that can be an indicator of ischemic condition of the respective tissue. Ischemic conditions can be caused by heart failure, shock, suffocation, intoxication, carbon monoxide, and respiratory insufficiency [11]. It can also play an important role in sports medicine and is able to monitor athletes during exercise [11]. When muscle fatigue occurs, it is due to accumulation of lactate in working muscles which cause inhibition of contractile processes. During exercise, lactate concentrations can increase up to 40 mmol/L (mM) in the muscle fibers [93]. A key performance indicator for endurance athletes is lactate threshold. Lactate threshold allows for the athlete to perform at the highest rate of work for extended periods [94]. The lactate threshold is defined as the exercise intensity in which lactate starts to accumulate in the bloodstream. This occurs when the body is unable to metabolize the lactate that is produced during exercise [95].

Blood

Blood has been used since 1886 to measure lactate levels. In 1964, Broder and Weil proposed that lactate was a prognostic biomarker [96]. They observed if there was excess amount of lactate levels, then it was associated with poor outcome in patients [86]. There is a relationship between increased blood lactate levels and tissue hypoxia in patients. Tissue hypoxia is when there is lower than normal oxygen levels in the tissue and the oxygen delivery is not being met. Lactate levels in blood will rise when tissue hypoxia occurs [96]. Normal levels of lactate in blood is around 0.5 mM. When lactate levels increase in blood exceeding 7 - 8 mM this can be associated with fatal outcomes. Lactate is a major indicator of ischemic conditions [97].

Lactate blood sensors are invasive sensors than can be implanted in the body and continuously monitor lactate. Implantable sensors can be used in hospitalized patients. The design

of implantable sensors is the longevity, since it will be *in vivo*. Implantable sensors will allow for monitoring lactate acidosis, acute circulatory shock, heart disease, and continuous monitoring in surgery [98]. Guiseppi-Elie et al., designed and fabricated an implantable biochip for dual monitoring of glucose and lactate [99]. The implantable sensors allow for continuous monitoring of dual analytes and was able detect up to 90 mM of lactate. The implantable sensor did show a decline in the activity of the enzyme after three months. This sensor was stored at 4°C, which is below the internal body temperature, and the enzymes on the electrode could decline quicker if in a warmer environment. When temperature increases, so does the enzyme activity to a certain point and then the enzyme will start to denature [100]. When the sensor is implanted into the body, it is exposed to flowing whole blood, where plasma proteins adsorb onto the surface of the device. This then leads to adhesion activation and aggregation of platelets that trap red blood cells, which will form a blood clot. This will affect the surface of the device due to restricting the mass transport of analytes to the sensing surface (as shown in Figure 11) [101].

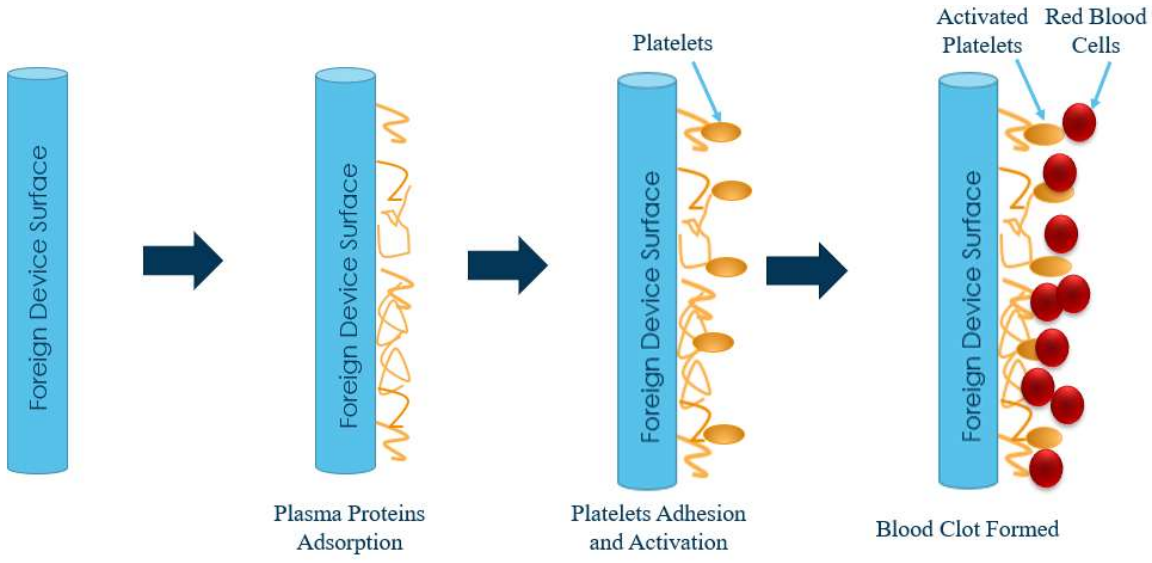

Figure 11. Schematic of an implantable sensor. Adapted from K. H. Cha et.al [101]

Overall, this can lead to deterioration in sensors performance and response [101]. Another disadvantage of an implantable sensor is it requires a physician to surgically insert the implant into the patient [103]. The sensor is *in vivo*, which can lead to bacterial infection from the sensor being implanted into the human body.

Sweat

An alternative to invasive implantable sensors is non-invasive sweat sensors that can be placed on the body without surgery. Sweat plays a critical role for human thermoregulation which can be due to environment or exercise-heat stress. There are three main type of sweat glands: eccrine, apocrine and apoecrrine (as shown in Figure 12) [103].

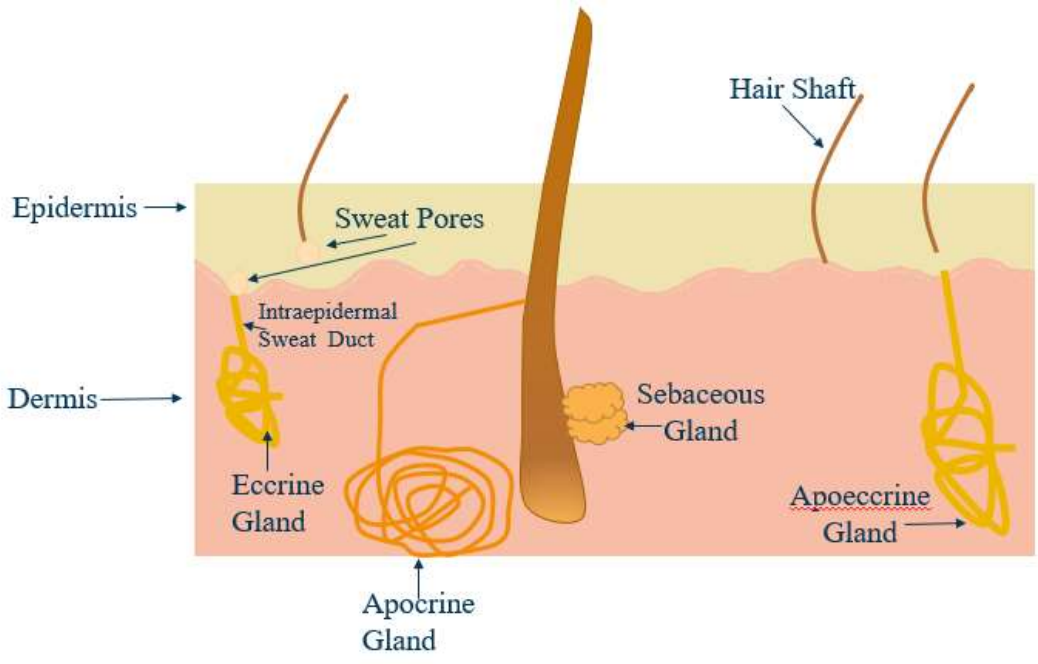

Figure 12. Schematic of the structure of the human sweat gland. Adapted from L. B. Baker [103]

The eccrine gland was first described in 1833 by Purkinje and Wendt and in 1834 by Breschet and Roussel de Vouzzem. The eccrine gland was named 100 years later by Schiefferdecker. Humans have 2 - 4 million eccrine sweat glands, which are found in palms, soles, and non-glabrous skin. The palms and soles have the highest gland densities and respond to emotional and thermal stimuli. The apocrine gland was first recognized in 1844 by Krause and then named by Schiefferdecker in 1922. This sweat gland is primarily found in the axilla, breasts, face, and scalp. These glands are larger and open into hair follicles. In addition, these glands produce lipid rich sweat which consists of protein, sugars, and ammonia. The apocrine sweat glands respond to adrenergic and cholinergic stimuli. The third gland is apoeccrine gland which was first described in 1987 by Sato. This gland was developed from the eccrine sweat gland from the ages of 8 to 14 years old. The apoeccrine glands are located only in the axillary region. This gland produces copious saltwater secretions. This also responds to cholinergic than adrenergic stimuli [103]. Sweating is initiated by the hypothalamus in response to an increased rise in temperature of the body. Then a signal is sent by the sympathetic nervous system. Sweating is stimulated *in vivo* and *in vitro* using both α-adrenergic and β-adrenergic agonists [104]. Sweat contains mainly ions such as sodium, potassium, magnesium, chloride and lactate. The upper duct is also known as the acrosyringium. This region consists or straight and coiled portion in the dermal duct. The eccrine sweat gland expands and then emerges to surface of the skin. The microfluids of the eccrine sweat gland are very important for biomarkers with strong sweat rate dependence.

The benefit of using sweat is that it is easily accessible and noninvasive [105]. Sweating can occur in physical responses to environmental conditions, physical exercise or a person's emotions [104]. Sweat lactate can track an individual's performance or exertion levels [106]. This can be used for athletes and measure their lactate threshold. Lactate threshold allows athletes to

perform at a higher work-rate for extended periods [107]. Lactate threshold indicates when an individual is at the point of intensity when the blood lactate concentration begins to exponentially increase. The individual or athlete will need to immediately reduce the intensity so overstraining does not occur [108]. When a person becomes ill, the illness can change the composition of their sweat, which can alter certain components in sweat. These components then can act as a biomarker [109]. Work presented in this paper can detect lactate in sweat, which is a noninvasive alternative to blood. This sensor can detect sweat lactate concentrations over lactate threshold, which can be indicator for lactic acidosis and ischemic conditions. Sweat lactate can be used as a biomarker for tissue viability and for pressure ischemia. Also, it can be indicators for cystic fibrosis (CF), electrolyte imbalance, physical stress, osteoporosis, bone mineral loss, sodium, lactate, and ammonium levels. It can also be used for monitoring a person's intoxication level and drug abuse [26]. Sweat concentration of lactate and ammonium ion are about 10 to 100 times higher than blood, which allows it to be easily detected using a biosensor [110]. During exercise lactate concentrations are around 31 mM compared with 12.1 mM at the point of exhaustion [104]. Recently, Payne et al., designed a flexible lactate sensor that detects lactate in the sweat [111]. This sensor can detect up to 24 mM of lactate, whereas the sensor we developed can detect pass the threshold lactate concentration of sweat during exercise.

The benefit of using sweat to detect lactate is that it is convenient, accessible and it is correlated to lactate levels in blood [105]. To collect information on athletes' physical condition an insertion of a catheter or implantable biosensor would have to be used, this can lead to infection and it is not convenient to the athlete for safe and convenient monitoring lactate.

CHAPTER 4

LACTATE BIOSENSOR

Current Trends and Outlooks

Lactate concentrations have not only been used for clinical diagnostics for assessing patient's health conditions, sports medicine, shock/trauma, but also in the food industry. In the food industry, lactate can determine the presence of bacterial fermentation which indicates the quality and freshness of the food, whereas in the sports medicine field, lactate levels are used for determining physical fitness in athletes [82].

Other analytical methods that have been used for lactate determination are high-performance liquid chromatography, fluorometry, colorimetric test, chemiluminescence, and magnetic resonance spectroscopy. The drawbacks of these methods are that they are time consuming and costly due to the requirement of the machinery and trained manpower. Using biosensors can eliminate some of these limitations. Biosensors allow for real time data acquisition, rapid response, and they are user friendly and economical. The most common lactate biosensors are enzyme lactate sensors due to their low detection limit, sensitivity, comparatively simple fabrication, user friendliness, portability, reliability, and reasonable cost [82]. Current trends of lactate sensors are noninvasive applications, such as monitoring in saliva [112], sweat[105], and tears[113].

Monitoring lactate through sweat has shown to be the most convenient for evaluating physical performance [114]. Sweat has been shown to be the most promising biofluid to measure lactate. This is due to sweat being easily accessible to collect and offers physiological information.

Sweat lactate has been shown to be a good biomarker for evaluating the severity of peripheral occlusive arterial diseases and damage in soft tissue [115]. Noninvasive lactate biosensors are also incorporated into biofuel cell, where the biofuel cells harvest energy from metabolites present in various biofluids. The metabolites is utilized as a potential fuel for energy [116]. In human perspiration, there is high levels of lactate concentrations which can be used to power the device [117].

Materials

Buckypaper is composed of a dense network of multi-walled carbon nanotubes (MWNCTs) was bought from NanotechLabs (Yadkinville, NC, USA). Pyrenebutanoic acid succinimidyl ester (PBSE) was purchased from AnaSpec Inc. Lactic acid, potassium phosphate monobasic, dimethyl sulfoxide (DMSO), isopropyl alcohol and d-lactate dehydrogenase (D-LDH) were acquired from Sigma Aldrich and were without further purification. PDMS was obtained from Dow Corning (Midland, MI, USA). Phosphate buffer and phosphate-buffered saline (PBS) were prepared with 18.2 MΩ·cm Milli-Q water.

Working Electrode Fabrication and Design

To make a small-scale sensor while maintaining high sensitivity, the BP was cut into a circle with a diameter of 9 mm. Then 200 μm tungsten wire was sandwiched and sealed along the top edge of the circle using polyimide and an additional 2 mm × 2 mm strip of BP. The polyimide was applied around the outside edges of the circle to enhance the structure of the BP. The bioelectrode was cured at 100 °C for fifteen minutes. This is shown in Figure 13.

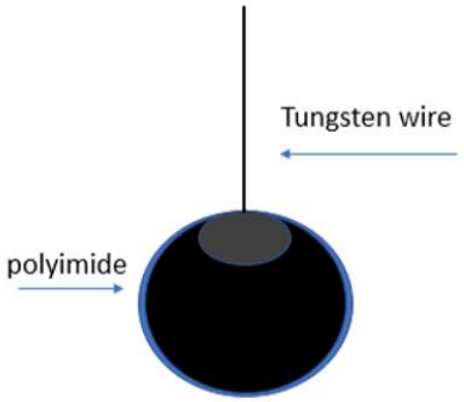

Figure 13. Schematic of the fabricated working electrode.

The electrode was washed with 2-propanol for fifteen minutes to remove any impurities on the surface. The circular BP was then placed in 1.4 mg/ml PBSE/DMSO cross-linking solution and was done in the dark for one hour with moderate shaking. Next, the electrode was rinsed with DMSO for five minutes and followed by another rinse of 10 mM of phosphate buffered solution (PBS) (pH 7.0) for five minutes to remove any leftover DMSO. Afterwards, a solution of 1.4 mg/ml of D-LDH prepared in 10 mM PBS (pH 7.4) used for enzyme immobilization. Next, 50 µL was drop casted on the front after being prepared in the dark at room temperature with moderate shaking for an hour. Then the electrode was turned over and 50 µL was drop casted on the other side following the same procedures as outlined previously. Then coated the bioelectrode with 5 µl of Nafion on the surface area. Nafion is used on the surface of the electrode because it is biocompatible to the enzymes and is chemically inert, which entails it exhibits relatively little adsorption of species from the solution. Nafion reduces the effects of electroactive interferents such as ascorbate. It is also used as a protective membrane for the biosensor [118]. Nafion was chosen over chitosan because it is more conductive and over time Nafion enhances the electrode stability of the electrode [119]. Next, the bioelectrode was dried in the desiccator at room

temperature for fifteen minutes. This allows for the immobilization of D-LDH to be preserved on the bioelectrode. The electrode was placed in a sealed container for 24 hours until tested.

PDMS film was prepared using PDMS monomer and the curing agent with a mixed ratio of 10:1 and cured in the oven at 50 °C for ten minutes (shown in Figure 14). Next, a hole was punched in the center of the PDMS and the working electrode was affixed. Then, the counter and working electrode was weaved through the PDMS (Figure 15). Small amounts of PDMS were placed to enclose the holes of the working, counter, and reference. Finally, the PDMS was placed on athletic tape for adhesion (Figure 16).

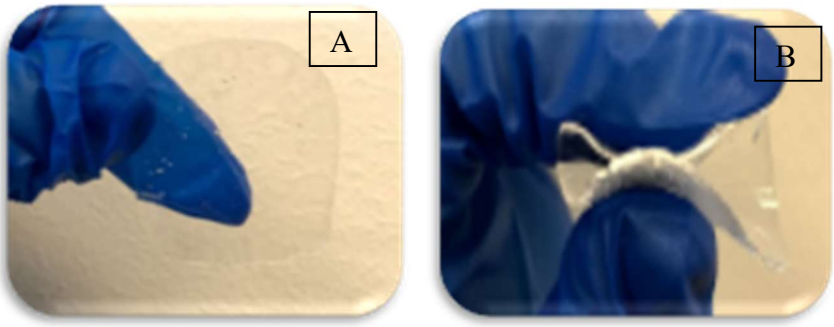

Figure 14. (A) PDMS cut into a square and (B) PDMS bent

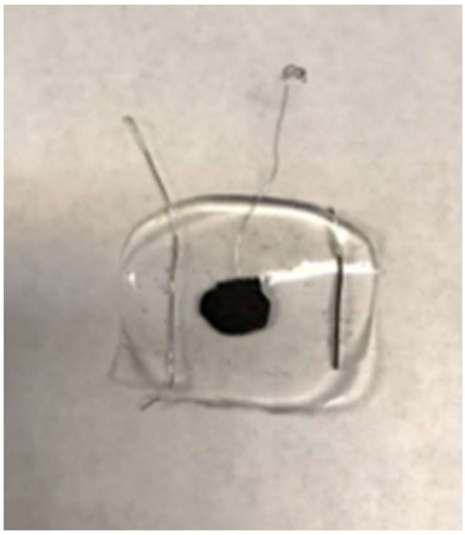

Figure 15. Fabricated lactate biosensor prototype

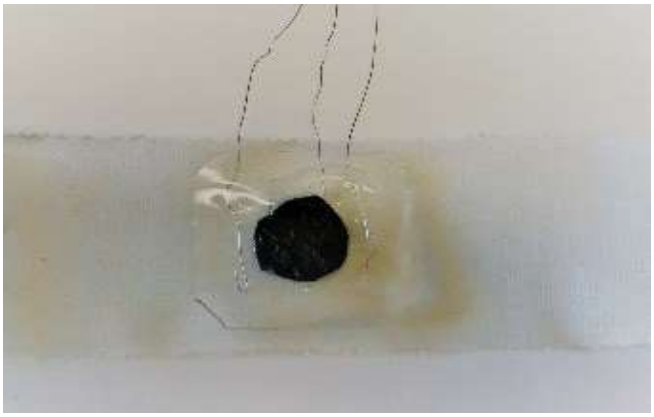

Figure 16. Lactate biosensor prototype incorporating athletic tape to enable facile attachment to skin

Reference and Counter Electrode Fabrication

A 20 mm long and 1 mm thick silver (Ag) wire is placed briefly in 0.1M HNO_3 to remove any oxide layer on the surface of the wire. Then, the silver wire is rinsed with DI water, IPA and dried in a N_2 gas. Next, the Ag wire electrode is oxidized in 3 M NaCl at +700 mV applied voltage using chronoamperometry techniques. This resulted in the formation of a white coating of AgCl on the surface of the electrode. The electrode was then rinsed with and stored in 3 M KCl. The counter electrode is a 20 mm long platinum wire.

Results

The working electrode material is made from a mesh network of multiwalled carbon nanotubes (MWCNT's). MWCNTs are a type of carbon nanotubes (CNTs). CNTS are rolled up sheets of graphene that exists as a hollow tube [120]. This material has shown to be a promising material for sensing application. CNTs have an outstanding ability to mediate fast electron transfer kinetics for a wide range of electroactive species. CNT chemical functionalization can be used to attach almost any chemical species to them. This enhances the solubility and biocompatibility of the tubes [121]. For use in the design of the sensor, the MWCNT's were cut into a circular shape. This was chosen to ensure uniform distribution of enzyme solution on the surface of the bioelectrode. D-LDH enzyme is used to catalyze the oxidation of lactate [122]. The initial reaction that creates the enzyme immobilization reaction is derived from the creation of a peptide bond between the amino group from the enzyme and the carboxyl group which is found within 1-Pyrenebtanoic acid, succinimidyl ester (PBSE), a heterobifunctional crosslinker. As this reaction occurs, the double bond between the carbon and oxygen is broken off from the PBSE. This causes oxygen to have a negative formal charge with the electrons moving towards the electron with a higher electron count. Because of this, the carbon becomes electron deficient which leads nitrogen

to donate electrons creating an unstable intermediate with a now positive formal charge nitrogen and oxygen retaining its negative formal charge. The oxygen is then able to reform a double bond with the carbon atom on the PBSE using the π bond electrons from the oxygen atom. This happens concurrently with the ester group, that was previously attached to the carbon atom, breaking off along with the hydrogen that was previously attached to the nitrogen. The result of this reaction leaves behind a neutral, immobilized enzyme (as shown in Figure 17) [85, 86].

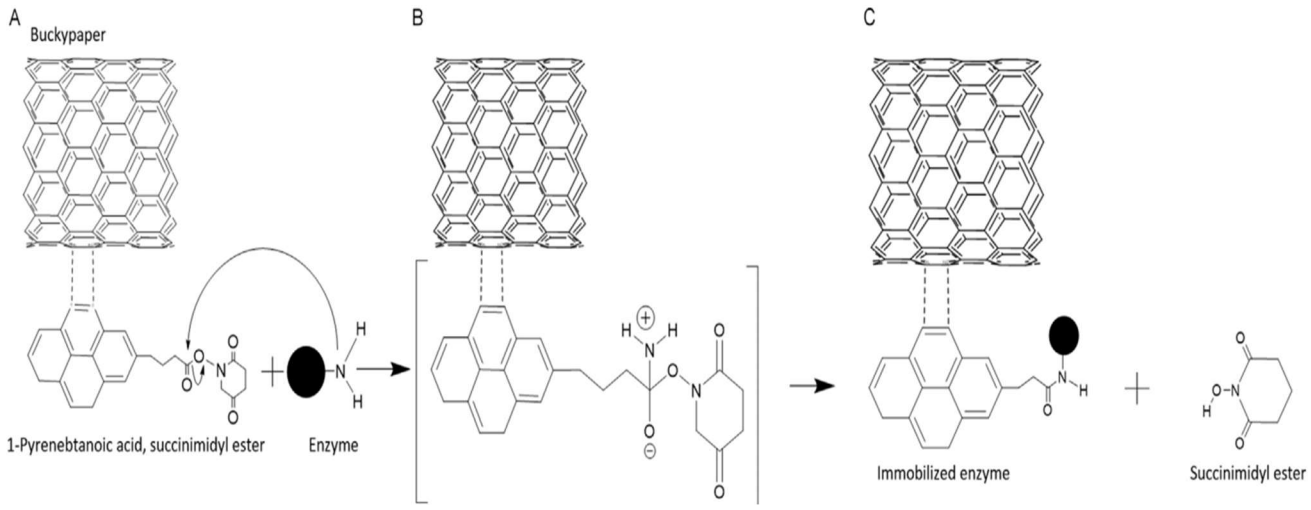

Figure 17. Schematic of the enzyme immobilization. (A) PBSE interacting with MWCNT's via π-π stacking and amino functional group on the enzyme reacts with the carboxyl functional group on the PBSE to form a peptide bond. (B) Formation of an unstable intermediate product. (C) Immobilized enzyme along with the byproduct. From T.Kulkarni et.al [123] copyright line © [2017] IEEE

After two months, the electrodes were weaved into PDMS, a polymer that is used for fabrication for flexible microelectronics [124]. This is one of the most common polymer materials used in biomedical applications due its ease for fabrication, biocompatibility, and haemocompatible [125]. PDMS has also been used as a biomaterial in catheters, drainage tubing,

insulation for pacemakers, membrane oxygenators, and ear and nose implants [126]. The benefits of using PDMS are it is inexpensive, flexible, and non-toxic to cells. In addition, this is a soft flexible substrate, optically transparent, and very thin [124]. This will allow for the sensor to be placed on the body.

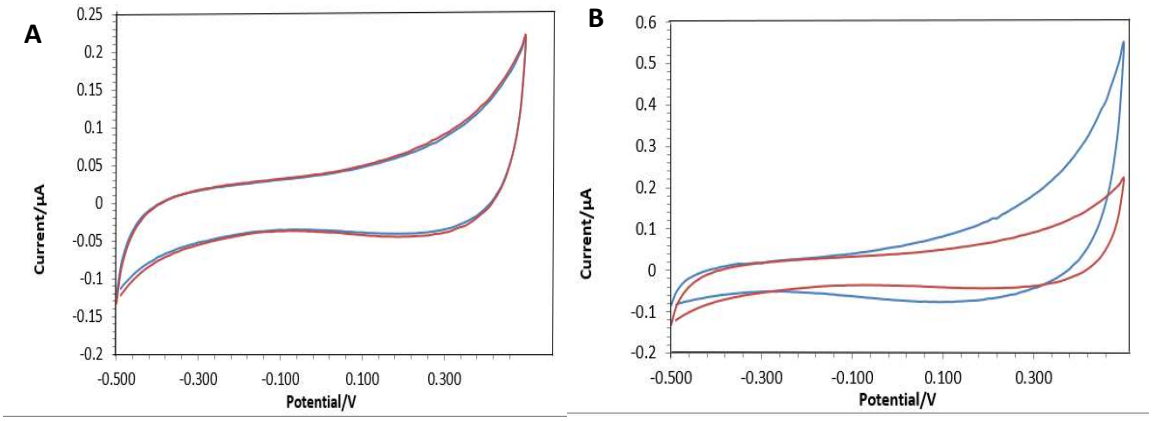

Figure 18. (A) Pt wire electrode (red curve) in 10 mM phosphate buffer solution. (B) As-fabricated Ag/AgCl reference electrode (blue curve) and commercially available glassy carbon electrode, Ag/AgCl and Pt wire were used as the working, reference and counter electrodes.

Cyclic voltammetry (CV) and chronoamperometry (CA) were performed with the PalmSens4 in order to electrochemically characterize the biosensor. All experiments that were conducted were done in triplicates. The setup of the biosensor system, which consists of platinum counter, BP/D-LDH working, and Ag/AgCl reference electrodes. Figure 11 shows the prototype biosensor, which incorporates an athletic tape to affix the biosensor to the skin. Figure 18 shows the CV curve for the platinum (Pt) wire counter electrode versus the commercial Pt electrode which showed no significant potential difference observed between the two counter electrodes. In addition, the as fabricated reference electrode showed an increase in the oxidation compared to the

commercial Ag/AgCl, which is within in the experimental error. The electrocatalytic activity of the biosensor was assessed using cyclic voltammetry in the presence of 10 mM phosphate buffer solution (pH 7.4) and in the presence of the increasing concentration of lactic acid. A well-defined oxidation peak was observed at a potential of 0.15 V as shown in Figure 14.

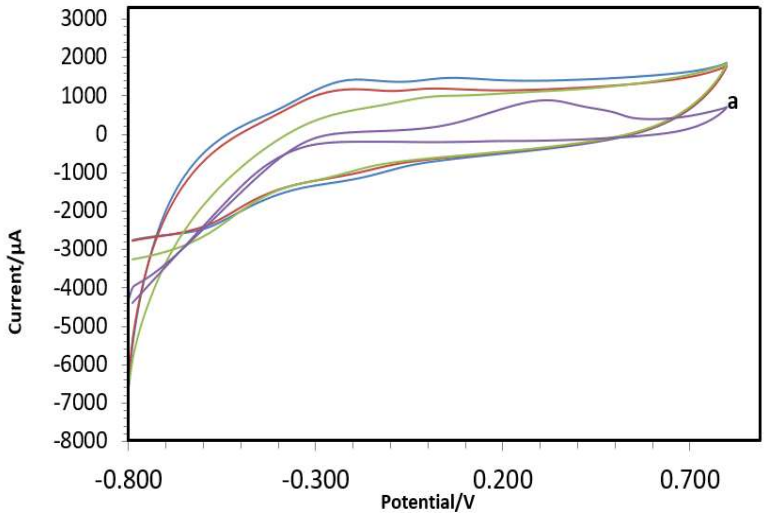

Figure 19. Cyclic voltammetry of the lactate biosensor in the absence and presence of increasing lactic acid (1, 20, and 40 mM). (a) in the absence of D-LDH.

In chronoamperometry, the potential of the working electrode is stepped from a value which no faradaic reaction occurs to a potential at which the surface concentration of the electroactive species becomes zero. It is used by pulsing the potential of the electrode repetitively at fixed time intervals [34]. Chronoamperometry is used to measure current–time dependence for the diffusion of lactic acid added in phosphate buffer solution. In response to the addition of 5 mM lactic acid aliquot, there was a clear increase in current, which indicated that the lactate biosensor is responsive to lactic acid at an impressed potential of 150 mV (Figure 20). This potential was chosen to match the oxidation peak observed in the CV (Figure 19) for the oxidation of lactic acid.

Lactate oxidase produces hydrogen peroxide which will cause the enzymatic reaction to have a higher oxidation potential. Multiple lactate biosensor that used lactate oxidase had an applied potential of +650 mV [16,108]. This could cause electro-oxidizable species, such as ascorbate to be easily detected thereby interfering with the biosensor response to lactic acid [82, 115]. In this work we used D-LDH in order to maintain the high selectivity of the biosensor.

Anastasova et al., [105] developed a wearable multi-sensing patch that was only able to detect a linear range up to 28 mM of lactic acid. Whereas in the work we observed a linear range up to 45 mM of lactic acid (shown in in Figure 20), which is above the average exercise sweat lactate concentration of 31 mM [104]. This indicates this sensor would be able to monitor ischemic conditions and other physiological conditions that elevate lactate levels. This sensor can be used in surgery or organ preservation to monitor lactate levels during surgery and be placed on the tissue or organ to monitor lactate levels [102]. Increased levels of lactate in blood around 7 - 8 mM can be caused by underlying health conditions [98]. This sensor can measure lactate levels from 2 - 45mM. This indicates it would be able to detect lactate blood levels that are at dangerous levels.

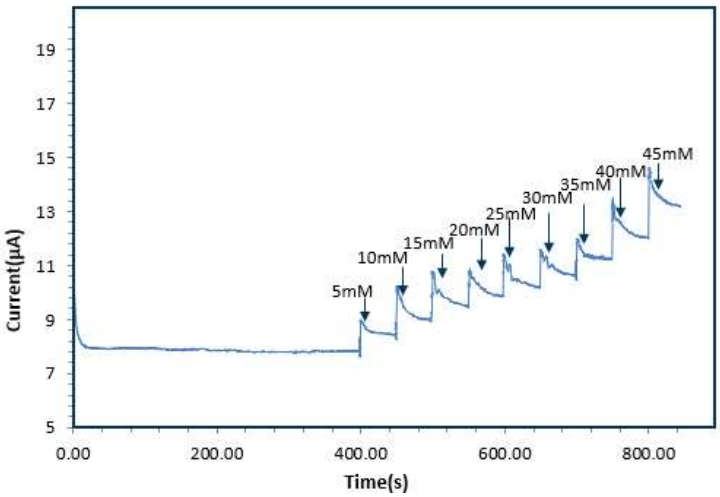

Figure 20. Current responses of CA Day 1 MWCNTs /D-LDH/nafion electrodes in PBS to the successive injection different concentration of lactic acid at 0.15 V, n=3

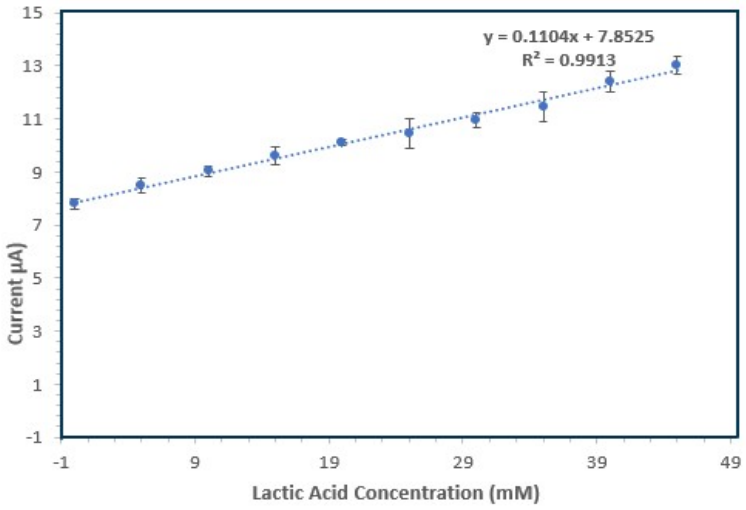

Figure 21. Calibration plot of the response of lactate biosensor, n=3

Due to the biosensor being placed on the skin, a pH and temperature profile characterization was conducted. Figure 22 shows the optimal working temperature of the biosensor operating at a fixed pH of 7.5 is 50 °C. When a person begins to exercise, an increase in blood flow will occur which will then increase the temperature of the skin [127]. As shown in Figure 22 the temperature increases as well as the current. Figure 23 shows that the biosensor works better in a more acidic or basic environment. During exercise the pH will increase on the surface of the skin making it closer to a pH of 7 which is a more neutral environment [128].

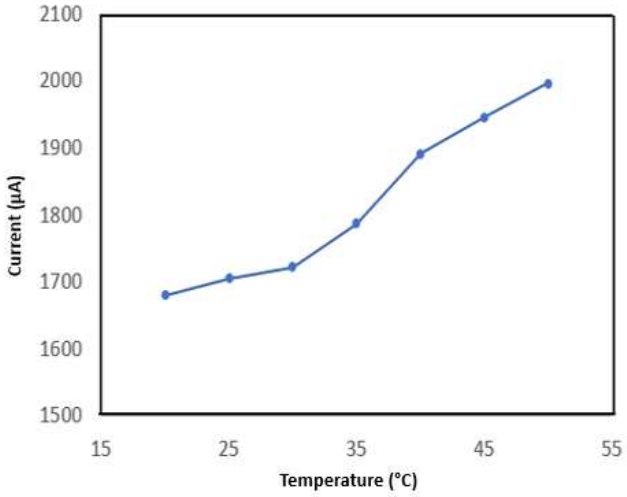

Figure 22. Temperature dependence on MWCNTs /D-LDH/nafion in the presence of 5mM of lactic acid

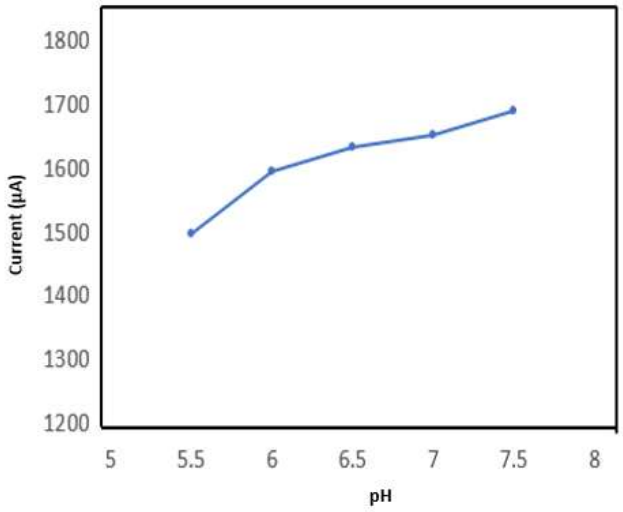

Figure 23. pH dependence on MWCNTs /D-LDH/nafion in the presence of 5 mM of lactic acid

Once the working electrode was calibrated, the system was fully integrated together. The working, reference, and counter electrode were placed into PDMS after 60 days and then the system was connected to the PalmSens (shown in Figure 34). A chronoamperometry measurement was performed of the completed sensor. Figure 25 shows a linear increase in current in response to 5 mM lactic acid aliquot at an applied potential of 150 mV. The sensitivity of this sensor is 0.679 μA/mMcm^2. This sensitivity is relatively close to the working sensor without PDMS, which indicates it can be used to monitor lactic acid.

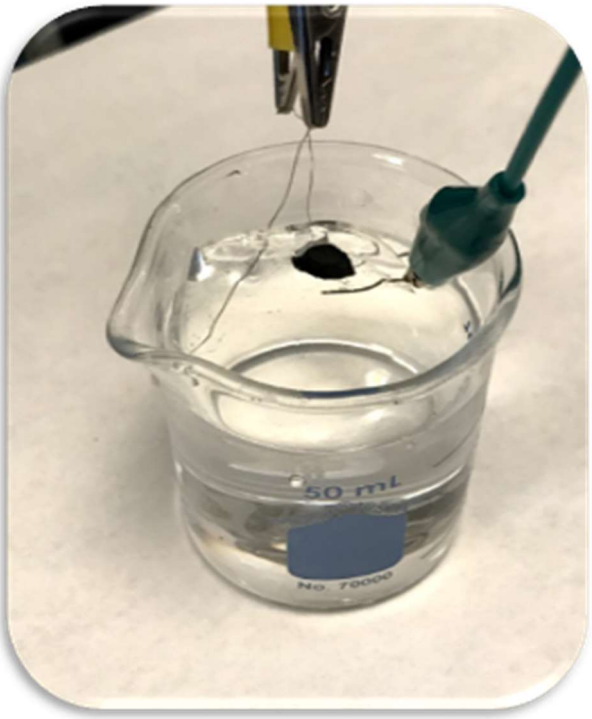

Figure 24. Working, reference and counter electrode integrate into PDMS after two months and placed in PBS.

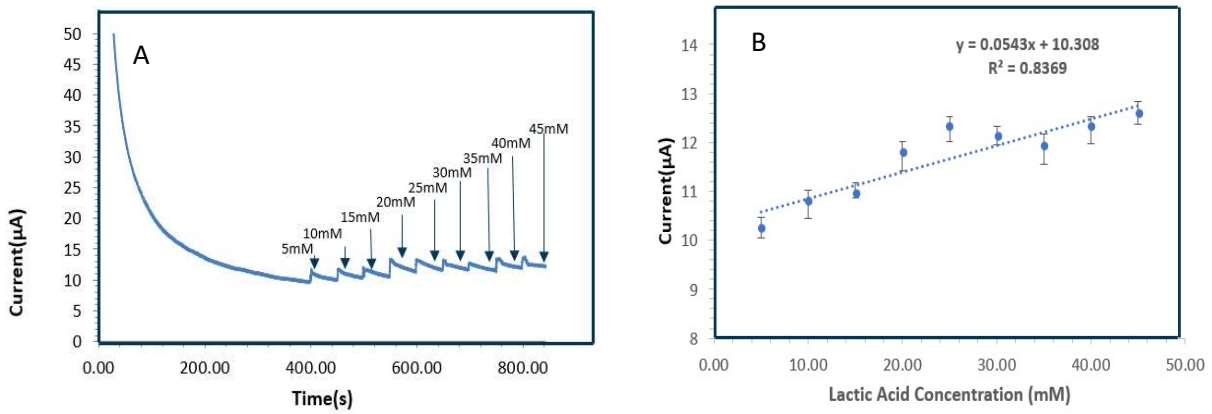

Figure 25. (A) Current responses of CA Day 60 MWCNTs /D-LDH/nafion on electrodes on PDMS in PBS to the successive injection different concentration of lactic acid at 0.15 V, n=3 (B) Calibration plot of the lactate biosensor, n=3

The lower sensitivity of 0.679 μA/mMcm^2 was anticipated since upon complete sensor integration, the PDMS covered half the working electrode surface. This is believed to have led to the observed decrease in sensitivity. Additionally, a decrease in sensitivity is also expected over two months of storage as the enzyme activity degrades over time.

CHAPTER 5

CONCLUSION

A flexible electrochemical lactate sensor that is sensitive to the detection of lactic acid and has been successfully designed and fabricated by immobilizing D-LDH on a mesh network of MWCNTs. The lactate dehydrogenase enzyme exhibited a high electrocatalytic activity in the presence of lactic acid. This sensor exhibited an excellent range of up to 45 mM for lactic acid and a sensitivity of 1.388 $\mu A/mMcm^2$. The lactate biosensor also showed stable operation at physiological pH and temperature, which demonstrates that the sensor could monitor sweat in different operating environments. Upon complete integration of the biosensor system and lower sensitivity is observed. This could have been examined by performing a stability test on day 1 to ensure the decrease in sensitivity was caused by the working electrode being covered by half of the PDMS.

Lactic acid is a key biomarker for lactic acidosis that can cause weakness and fatigue in the muscles. During intense and extraneous exercise, lactic acid will increase in the body. When lactic acid increases, the liver will typically break down the lactic acid. The biosensors is fabricated on a flexible PDMS to help predict intensive care, training status and fitness during exercise. It has been shown that this type of biosensor has a practical application in clinical and sports medicine. This sensor could be used on a wide scale basis to help improve the health of consumers due to continuously monitoring lactate levels while being active.

Future Work

The future work involves making more stable biosensors than can be stable under continuous operations. Since the sensor was not tested on day one when integrated into PDMS, this will need to be conducted to see if the PDMS or the duration of storage time impacted the sensitivity. The sensor will need to go from day one and measured weekly for two months on PDMS to determines its stability and sensitivity on the substrate. The sensor will need to be tested on the athletic tape to indicate that it has similar results to the working electrode and the working electrode placed on PDMS. Then the sensor can be tested using human sweat. Once this has been conducted, the sensor can be tested on a person during exercise to monitor the ability of the sensor.

Additional stability, selectivity and strength test will need to be performed. The stability characteristics that will be tested are the shelf life, reusability, and continuous use stability. Since this biosensor does contain biological components, the stability test will provide data on the duration the enzymes can stay on the surface and detect lactic acid. Then a selectivity test will test for common interfering species such as glucose, ascorbic acid, and uric acid.

Since this is a flexible sensor, a mechanical distortion (pinching, twisting, and stretching) test needs to be conducted on the integrated sensor and PDMS. The sensor can integrate wireless data acquisition, where the data obtained from sensor can be wirelessly transferred to a mobile device through Bluetooth where it can be further studied and analyzed. Lastly, the sensor could be integrated into a wearable biofuel cell for detecting lactate through sweat in the development of a self-powered lactate biosensor. This would allow the system to harness lactate in sweat as an energy source and produce enough levels of electricity to power the system.

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