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LACTATE BIOSENSORS FOR CONTINUOUS MONITORING

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1. ABSTRACT

Blood lactate concentration is a highly sensitive measure of tissue oxygen deprivation from ischemia, trauma, and hemorrhage, which can produce life-threatening shock. Significantly, blood lactate is the most reliable end point indicator of resuscitation and predictor of survivability. The need for continuous measurement of blood lactate, and the slowness of discrete conventional photometric assays, is leading to the development of monitoring systems based on electrochemical lactate biosensors. Research and development of both *in vivo* and *ex vivo* biosensor systems is ongoing. *Ex vivo* biosensors are used with implanted microdialysis or ultrafiltration probes. Lactate from the blood diffuses into the dialysate/filtrate is transported outside of the body for measurement. The external biosensors are protected from fouling or contamination from unwanted blood constituents, but at the cost of an inherent delay in response despite system miniaturization. *In vivo* biosensors provide a direct measurement of blood lactate concentration, providing rapid response to changes in lactate levels. *In vivo* sensors are placed in the skin or implanted subcutaneously. Response to changes in lactate concentration is rapid, but biocompatibility requirements are more stringent than for *ex vivo* sensors. As is the case with all microdialysis systems, some *in vivo* biosensors must be implanted into the body using an insertion needle or surgical means, limiting their use. However, small, inexpensive, disposable *in vivo* sensors are also being developed which can emplaced and removed by the user.

2. INTRODUCTION

A biosensor contains a biological molecule that senses the presence of a specific analyte, producing a biological change that is converted by a transducer component into a measurable output, such as an electrical or optical signal. Biological sensing agents are typically enzymes, antibodies, or microorganisms, but DNA and other biological molecules can be used. Lactate biosensors use enzymes as the biological sensing component.

Biosensors hold the promise of rapid, accurate detection and quantification of a specific analyte of interest at the point of care, or site of origination of the analyte. In contrast to conventional clinical diagnostics, sample storage and transportation are avoided, and analytical results are rapidly available. Assay costs are reduced to the point that a sensor can be used once and then discarded. Medical biodiagnostics represents the largest global market sector for biosensors, 92% in 1996 according to a study published by Cranfield University (1). However, diabetic glucose monitoring overwhelmingly dominates the medical diagnostics market sector because of the prevalence and seriousness of the disease and the improvement in disease control achieved by frequent (ideally continuous) glucose monitoring. Biosensor use for analytes of medical interest other than glucose is relatively very small, 2% in 1996 (1), and thus represents an emerging market.

Lactate biosensors are used in some benchtop clinical laboratory instruments, competing with older (and slower) photometric methods. They are also used commercially in benchtop instruments to measure lactic acid (produced by lactose fermentation) in milk and other foods. Three manufacturers, Roche Diagnostics (Germany) and Arkray (Japan) and Senslab (Germany) manufacture small handheld biosensor-based lactate meters directed primarily to sports medicine applications. The Roche Accusport/Accutrend uses a 20 microliter sample with an optical readout, the Arkray Lactate Pro uses a 5 microliter blood sample with an electrochemical readout, and the recently-introduced Senslab Lactate Scout requires only a 0.5 microliter blood sample. All provide only intermittent "spot" lactate monitoring. A recent editorial by Klonoff

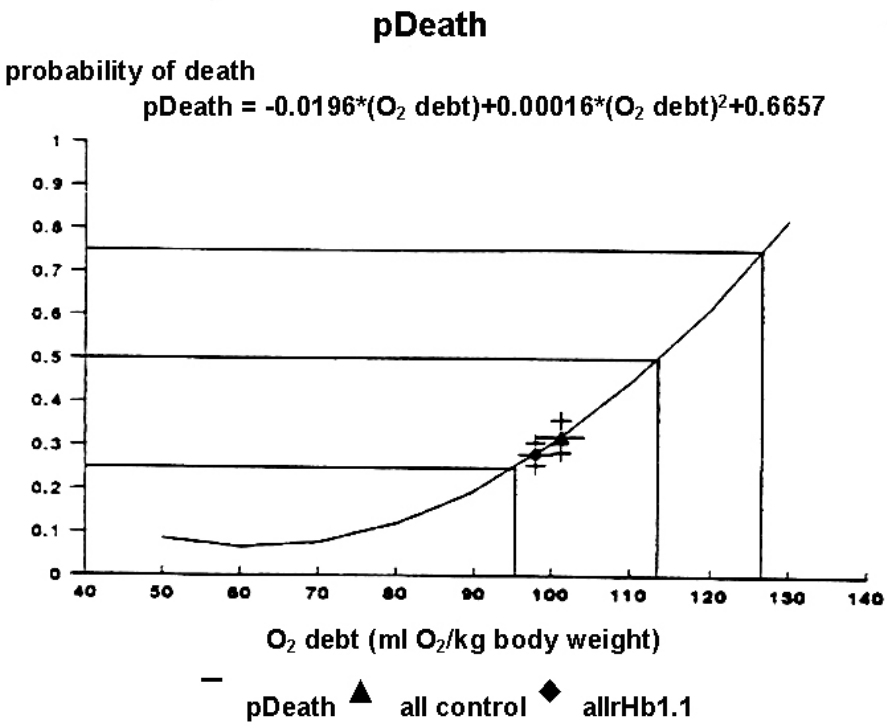

Figure 1. Kaplan-Meier regression function showing mean O_2 debt and probability of death. Resuscitation of canines with recombinant hemoglobin solution (rHb1.1) or colloid/blood (control).

discussed the need for both minimally-invasive and continuous lactate monitoring in biomedical applications and the current lack of such products (2). This review focuses on the development of biosensors for continuous monitoring of lactate in medical diagnostic applications.

3. SIGNIFICANCE OF BLOOD LACTATE CONCENTRATION

Elevated blood lactate is a clinically valuable diagnostic indicator for a number of medical conditions. Blood loss from trauma or during surgical operations can cause reduced circulation, leading to oxygen deprivation and shock, which can be defined as inadequate organ perfusion and tissue oxygenation (3). Respiratory failure and cardiac arrest can also reduce tissue oxygenation. As a result of inadequate tissue oxygen perfusion, mitochondrial respiration is inhibited and anaerobic metabolism occurs. Pyruvate, the primary cellular fuel, is diverted from its normal aerobic pathway and converted to lactate by lactate dehydrogenase. Aerobic metabolism in muscle tissue proceeds to pyruvate; however, under anaerobic conditions, lactate is formed because insufficient NAD^+ is present from the respiratory chain to sustain oxidation. Severe oxygen deprivation can produce lactate levels in excess of 30 mM, producing "lactic acidosis," leading to irreversible shock, if untreated. Thus, tissue blood lactate concentration is a good indicator of the extent of anaerobic metabolism, or equivalently, of tissue oxygen deprivation. Loss of blood, impairment of circulation, and the danger to the patient of shock can be quickly evaluated by measuring blood lactate (4-7).

The body possesses compensatory mechanisms which are capable of minimizing damage from inadequate oxygenation within limits, and thereby preventing the onset of shock. Compensation is a region of negative feedback able, for example during hemorrhage, to maintain arterial blood pressure by constricting the vessels, maintain adequate blood flow by increasing heart rate, increasing blood oxygenation by stimulating breathing rate, and maintaining renal blood flow by autoregulatory mechanisms within the kidney. However, if the limits of compensatory processes are exceeded because of severe trauma, massive blood loss, or prolongation of the injuries, shock decompensation ensues. Decompensation is a region of positive feedback or "vicious cycle," in which ischemic organs release abnormal peptides that affect whole body chemistries adversely, significantly in excess of the primary metabolic problems caused directly by the ischemia itself. Decompensated shock is a prelude to death.

Oxygen deprivation can be quantitatively expressed as oxygen debt, defined as the cumulative difference between baseline oxygen consumption and oxygen consumption during shock. The probability of death as a function of oxygen debt is shown in Figure 1, taken from reference 4.

Traditional parameters of perfusion (normal blood pressure, urine output, cardiac output and central venous pressure) are not sufficient indicators of the adequacy of resuscitation. Patients resuscitated to normal values of these conventional parameters still experience statistically excessive rates of organ failure and mortality

(4). The return of blood lactate concentration to normal levels, however, has been shown to provide an accurate endpoint of resuscitation in the critically ill patient (4-7). The ability to clear lactate to normal and the rate at which this clearance occurs together constitute the single most important variable predicting survival after severe injury. Continuous measurement of lactate levels is important to provide an accurate measure of this clearance rate. Other variables, such as base excess and anion gap, have been proposed as surrogates for lactate, but no consistent correlation between lactate and base excess or anion gap has been clinically observed (6). Lactate is the preferred measure of resuscitation endpoint and predictor of survival probability.

Substantial improvement in the survival of infants who underwent congenital heart surgery was achieved when their blood lactate level was monitored on a regular basis after surgery. By monitoring the infant's lactate level, detection of life-threatening oxygen deficiencies can be determined and corrected before damage becomes irreversible. In a recent study, reported by the Miami Children's Hospital, significant survival outcomes were observed in infants whose blood was tested for lactate levels every hour after surgery. The tests were conducted with a point-of-care, hand-held device that could produce a reading in two minutes requiring two drops of blood. Compared to national averages, the data showed a 74 percent decline in deaths in babies at highest risk, and a 63 percent drop in deaths overall. Dr. Anthony Rossi, Director of Cardiac Intensive Care at Miami Children's Hospital is quoted as saying, "The ability to use a tiny blood sample to accurately test lactate levels, a sensitive marker of oxygen debt, in two minutes at the bedside of an infant and then immediately adjust therapy is a major advance in our ability to make life-saving treatment decisions." He went on to say, "These are definitive and clinically significant findings that, for the first time, show how technological advances in blood testing directly and dramatically improve even the most fragile infant's chance of survival" (8).

As discussed above, an elevated concentration of blood lactate can indicate a variety of medical conditions, it can predict the probability of survival in subjects after open heart surgery, multiple organ failure, and septic shock (9-11). However, a clinical lactate measurement using conventional laboratory methods may take 30 minutes or longer, limiting its usefulness with injured or critically ill patients. A portable, handheld instrument to permit rapid measurement of blood lactate levels in the field or at the bedside (point of care), preferably on a continuous basis, is desirable for many of the more urgent medical applications and is the goal of significant research efforts.

Point-of-care monitoring of blood lactate is becoming more widely used in critical care as an early indicator of conditions such as acidosis or bacterial meningitis (12). Blood lactate is also used in the Intensive Care Unit to manage patients undergoing hemofiltration (13).

Measurement of blood lactate is also useful for exercise monitoring (14). Strenuous exercise can increase blood lactate levels from the normal value of 0.9 mM to approximately 12 mM. After cessation of exercise, the body recovers from this degree of anaerobic metabolism as the normal circulation reperfuses tissues and lactate is cleared by the liver.

4. LACTATE BIOSENSOR CHEMISTRIES

Four oxidoreductase enzymes have been studied for use in measuring lactate concentration. Three of these can use mediators as electron acceptors and one is relatively specific to molecular oxygen (15). The processes in three cases lead to pyruvate and in the other to acetate. Lactate dehydrogenase (16), cytochome b2 (17), and lactate oxidase (18,19) have all been used in lactate biosensor research. No reports of lactate-2-monooxidase use were found.

1. Lactate Dehydrogenase (EC 1.1.1.27) (from muscle tissue)

$$\text{L-lactate} + NAD^+ \longrightarrow \text{pyruvate} + NADH + H^+$$

$$\text{Half-cell: } NADH \longrightarrow NAD^+ + 2e^- + H^+$$

Enzymes utilizing the NAD^+/NADH couple (dehydrogenases) can participate only in two-electron transfers. Lactate Dehydrogenase from muscle tissue (EC 1.1.1.27) can also utilize NADP+, but it acts more slowly than NAD+. The irreversibility of the NAD^+/NADH couple at an electrode requires an additional catalyzed redox mediator step for electrochemical readout of this system.

2. Cytochrome b_2 (EC 1.1.2.3)

$$\text{L-lactate} + 2[Fe(CN^-)_6]^{3-} \longrightarrow \text{pyruvate} + 2H^+ + 2[Fe(CN^-)_6]^{4-}$$

At the electrode:

$$2[Fe(CN^-)_6]^{4-} \longrightarrow 2[Fe(CN^-)_6]^{3-} + 2e^-$$

Cytochrome b2 (EC 1.1.2.3) is identical with yeast lactate dehydrogenase. It is a hemoprotein with one flavin mononucleotide (FMN per heme). It catalyzes one or two electron transfers. The heme iron reversibly alternates between Fe(II) and Fe(III). There are two FMN's per enzyme molecule. It has very good ability to use electron acceptors other than O2.

3. Lactate Oxidase (EC 1.1.3.2)

$$\text{L-lactate} + O_2 \longrightarrow \text{pyruvate} + H_2O_2$$

At the electrode:

$$2H_2O_2 \longrightarrow O_2 + H_2O + 2e^-$$

Lactate Oxidase (EC 1.1.3.2) is an FAD flavoprotein which catalyzes a two-electron transfer. Many mediators have been shown to be effective. With a mediator, this system is analogous to the mediated glucose oxidase biosensor, with the major difference that the lactate/pyruvate equilibrium lies on the pyruvate side

whereas glucose oxidation goes to completion because of rapid non-enzymatic hydrolysis of the gluconolactone product to gluconic acid.

4.Lactate 2-Monooxidase (EC 1.13.12.4)
L-lactate + $1/2O_2$ $\longrightarrow$ acetate +CO_2 + H2O

At the electrode: Measure O_2 uptake

Lactate Monooxidase (EC 1.13.12.4) is an FMN flavoprotein capable of donating either one or two electrons. Molar mass = 260,000. There are two FMN groups per enzyme molecule. This enzyme shows poor ability to use electron acceptors other than oxygen.

5. CONTINUOUS LACTATE MONITORING

Continuous measurement of blood analytes is still at the forefront of biosensor research and development. Two primary approaches have been taken in developing biosensor systems for continuous monitoring of blood lactate levels:

- Implantation of a microdialysis probe to perfuse dialysate fluid into the body at a very slow fluid flow rate, using on-line biosensors to provide *ex vivo* measurement of lactate concentration in the dialysate outflow
- Implantation of lactate biosensors to provide *in vivo* monitoring.

Each approach has advantages and disadvantages. The microdialysis probe is considered to be more biocompatible and is easier to sterilize. The biosensor does not come into contact with the body tissues, and there is no possibility of the biosensor components leaching into the body or causing irritation. Because the dialysis membrane excludes large molecules such as proteins and blood corpuscles, no fouling of the biosensor can occur. On the other hand, there is an inevitable time lag due to the need to transport the dialysate stream to an external sensor for measurement. Because of the very slow flow rates that must be used to obtain reasonable analyte levels in the dialysate, this time lag is significant despite system miniaturization. Further, the equilibrium ratio between the concentration of the analyte in the body to that in the dialysate stream must be determined by separate calibration. The system is complex and the microdialysis probe must be implanted using an insertion set or surgical means. Microdialysis an excellent solution in a hospital or research setting where patient catheterization is common and several analytes may be monitored. A less invasive method would be desirable where only lactate is being monitored or in an ambulatory patient population. Microdialysis is more complex and therefore more expensive than an implanted biosensor.

An *in vivo* biosensor avoids the time lag required by microdialysis, providing a more immediate response. This rapid response is especially important where changing lactate levels must be detected rapidly to permit optimal medical treatment. Although currently, biosensors are implanted in the body using surgical implantation or a needle insertion set to penetrate the skin, the biosensor can be miniaturized and designed to permit self-insertion by the patient for subcutaneous placement. Lactate is a small molecule, and therefore subcutaneous interstitial fluid lactate levels equilibrate rapidly with levels in regional capillaries. A self-inserted biosensor will have significant advantages in ambulatory applications, avoiding the need for medically trained personnel. The sensor could also be removed and replaced at will, making a shorter (1 to 2 day) sensor lifetime practical if desired.

5.1. Continuous monitoring using microdialysis and *ex vivo* lactate biosensor

A microdialysis probe with a semi-permeable membrane is implanted in the biological tissue of interest and a dialysis fluid is flowed through the probe, perfusing into the surrounding tissue. The analyte to be measured diffuses into the dialysis fluid within the probe, which is then flowed into an external detection chamber of the microdialysis system at a very slow fluid flow rate. The microdialysis sample can then be analyzed by a variety of methods. Discrete samples can be measured by liquid chromatography or flow injection analysis. For continuous monitoring, an in-line biosensor or enzyme electrode system can be used.

A method of intercranial dialysis in rats was first reported in 1974 (20). Since that time, microdialysis systems have become available commercially for biological tissue and *in vivo* animal research studies. More recently, there has been growing interest in the use of microdialysis with *ex vivo* biosensors in clinical research and, ultimately, in clinical practice. Where microdialysis is already in hospital-based use as a clinical sampling method in cardiac or pediatric medicine, in neurological studies, and in intensive care, extension of the analytical technique to provide continuous monitoring provides an important benefit. Much of this work has been centered in Europe where the microdialysis technique first originated. There has been significant recent work on simultaneous lactate and glucose monitoring by Korf and co-workers in Gronigen, Netherlands (21-24) in collaboration with Jobst, Urban, Moser and co-workers in Freiburg, Germany (25-28) and Vadgama in the United Kingdom (22). The system has been successfully tested in human volunteers (29). An integrated microfluidics/biosensor chip was developed (30); this technology was spun-off by the University of Freiburg to Jobst Technologies in 2002. In earlier worker, Freaney and co-workers in Dublin, Ireland developed a total chemical analysis system (muTAS) chip with microdialysis sampling interface for application to lactate and glucose monitoring, and tested the system in dogs (31-33). Perdomo and coworkers, working in Muenster, Germany, also reported fabrication of a silicon microchip with integrated flow channels for lactate and glucose monitoring (34).

There has also been interest in continuous lactate monitoring using microdialysis with a biosensor in the United States and Japan. Kissinger and co-workers (35-36) reported the use of on-line lactate biosensors to

measure lactate concentration in microdialysis effluent, although much of their work on a variety of analytes has involved liquid chromatographic/tandem mass spectrometric analysis. Osbourne, Niwa, and Yamamoto (37) in Japan used a split-disk electrode geometry to measure glucose and lactate simultaneously.

Because of the medical and commercial importance of glucose monitoring, there has been considerable additional work on microdialysis/biosensor development for glucose alone. Although not involving lactate, instruments for continuous monitoring of glucose levels using microdialysis sampling and biosensor detection are nearing commercialization by Roche Diagnostics (Germany) and GlucoDay (Italy). Work on system miniaturization and integration may find application to lactate monitoring.

5.2. Continuous monitoring using *in vivo* biosensor

In vivo biosensors are preferable in terms of fast response, simplicity, portability and cost, and produce no biological fluid (dialysate) wastes. Certainly, *in vivo* implantable biosensors have to meet much more stringent performance requirements in terms of biocompatibility than biosensors used *ex vivo*. However, it is probable that biosensor fouling and leaching can be adequately minimized for a period of a few days to permit a stable biosensor signal. Chen (38) found that a "wired" enzyme glucose biosensor of the type developed by Heller maintained its *in vitro* sensitivity after implantation for four hours, and concluded that insertion-trauma-caused local perturbation had subsided sufficiently to permit reliance on readings. *In vivo* monitoring for relatively short periods of time (1-3 days) appears promising. Biosensor miniaturization is highly desirable to minimize tissue damage upon insertion or implantation and reduce consequent wound healing time. Where the lactate biosensor is to be placed within the dermal layer or subcutaneously, a biosensor system design permitting convenient and painless emplacement and replacement without assistance of professional personnel is highly desirable. More exacting requirements obtain for long-term monitoring (weeks to months), particularly where surgical implantation is required and it may be difficult to remove and replace the biosensor. Such placement would be required where localized changes in tissue lactate concentration must be measured rapidly at sites deep within the body. In such cases, tissue inflammation and reaction, clotting problems, and biosensor fouling must be minimized or avoided in order to achieve safe, stable and reliable long-term monitoring; no leaching of biosensor components must occur.

Most current work in Europe is focused on microdialysis systems for lactate monitoring. However, in addition to his collaboration on glucose and lactate monitoring of microdialysis effluent (22), Vagdama's research has included studies of biosensors for continuous measurement of blood lactate levels, primarily in flow systems using heparized double lumen catheters(39-42). His recent unpublished work has resulted in development of a biosensor for short-term continuous monitoring of blood lactate.

In North America, Heller has pioneered the development of "wired enzyme" biosensors that covalently bond both the enzyme and the enzyme mediator on a polymer backbone in an attempt to significantly increase the stability and sensitivity of the biosensor and to reduce interference (43). Although the major focus of the work has been on developing a "wired enzyme" biosensor for continuous monitoring of glucose, a number of other analytes have been explored, including significant work on lactate (44-49). Prior to its acquisition by Abbott Laboratories in April 2004, Therasense announced the development of a continuous glucose monitor based on Heller's work. The disposable, miniaturized "wired enzyme" sensor was designed to be easily inserted under the skin by the user using a spring-loaded insertion device. Much of this technology would be transferable to a continuous lactate monitor.

Wilson successfully developed a lactate biosensor used to monitor fluctuations of extracellular lactate levels in rat brain with simultaneous biosensor measurement of glucose and oxygen levels, permitting a comparison of changes in lactate and glucose levels under electrical stimulation (50). His 2000 review of the status of *in vivo* enzyme-based biosensor development included a discussion of lactate biosensors(51). Baker and Gough reported the development of an implantable lactate biosensor (52). In a submitted paper currently in review, Baker has discussed the results of implantation of a lactate sensor in dogs (53). Yang and coworkers have developed a needle-type lactate biosensor for continuous intravascular lactate monitoring and evaluated its performance in blood plasma (54). Continuous monitoring of blood lactate was reported by Marzouk and coworkers using a mediated lactate biosensor based on lactate oxidase with mediator (55-56). The biosensor was fabricated by packing the mediator in a cavity with dimensions of 1 x 2.5 x 1.5 millimeter and then immobilizing the enzyme on the mediator surface. The resultant biosensor was positioned in direct contact with a suspended muscle for monitoring lactate during ischemia.

Silicon microfabrication technology has been used to fabricate all-silicon microchips with integrated microneedles for painless blood glucose monitoring using an optical readout (57). Functional electrochemical lactate biosensors with integrated reference electrodes have now been incorporated into silicon microprobe chips. These microprobes, comparable in cross-section to a human hair, are capable of penetrating skin reliably and painlessly. Unpublished data from our laboratory indicate these miniaturized biosensors measure lactate concentration over the required physiological range. Continuous *in vivo* lactate monitoring in rabbits was successfully performed. The lactate microchip was positioned in a patch attached to the skin of the rabbit such that the biosensor-containing probe just penetrated the skin. The rabbit was then able to move freely in its cage during the monitoring period. No insertion needle or surgical implantation was required. *In vivo* biosensor outputs were recorded continuously and corresponding lactate concentrations determined from previous *in vitro* calibration. In addition, periodic blood

samples were drawn from each rabbit and analyzed by spectrophotometry. Calculated biosensor lactate concentrations correlated well with spectrophotometric results.

6. CONCLUSIONS AND PERSPECTIVE

Lactate biosensors are capable of meeting the need for continuous lactate monitoring in clinical chemistry. *Ex vivo* continuous monitoring appears promising although complex, assuming corrections for lactate dilution and partitioning can be adequately made and that the time lapse inherent even with system miniaturization can be tolerated. Microdialysis and ultrafiltration *ex vivo* systems are especially useful for hospital patients where catheterization is warranted and where multiple analytes are to be monitored. *In vivo* monitoring provides a more immediate response, and a smaller, simpler, inexpensive sensor. Assuming biosensor fouling problems can be resolved, *in vivo* biosensor monitoring is promising where lactate levels may change rapidly and for use outside of a hospital setting. The ability to easily and painlessly emplace and remove the sensor without medical assistance provides significant benefit in ambulatory use, particularly if the user is at a site remote from medical care.

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