Jurnal Teknologi, 54 (Sains & Kej.) Keluaran Khas, Jan. 2011: 47–59

ADAPTATION OF ON-LINE RAPID SAMPLING MICRODIALYSIS TO MONITOR HUMAN INTESTINAL ISCHAEMIA

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Abstract. Intestinal ischaemia or poor perfusion of the gastrointestinal tract is a major cause of post-operative mortality in abdominal surgery. Currently diagnosis of ischaemia relies only on clinical symptoms. Thus, monitoring bowel metabolism as an early marker of intestinal ischaemia is necessary. Human bowel microdialysis has been used in the past to study the metabolism of ichaemia collecting dialysate samples from the peritoneal cavity every 60 minutes. These sampling times carry a long delay for the detection of the typically rapid ischaemic event.

We have previously developed a successful biosensor system to monitor neurochemicals in the human brain during surgery and in the intensive care unit. The method consists of a flow injection analysis (FIA) system coupled to an enzyme based amperometric detector. The rapid sampling microdialysis monitoring system analysed electrochemically the dialysate glucose and lactate at high time resolution (typically 30 second sampling).

Adaptation of the analytical assay system for on-line microdialysis monitoring of human bowel was performed and validated for in vivo procedures. Optimum membranes loading ratios were found to be 1:0.5 GOx:HRP and 2:0.5 LOx:HRP. The ischemic range was found to be 15μM-400μM, 40μM-6mM for glucose and lactate, respectively. The calibration method for these monitorings was concluded with a range from 250 μM to 6 mM.

Keywords: Microdialysis; intestinal ischemia; biosensors; glucose; lactate

1.0 INTRODUCTION

Insufficient blood flow is the cause of ischemia, that leads to tissue injury due to

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the impairment of the tissue metabolic needs. Clinicians attempt to ensure both perfusion pressure and blood flow to avoid diseases [1]. Nevertheless, an early diagnostic is necessary to prevent further tissue damage when blood flow has been compromised.

Monitoring of gastrointestinal perfusion is limited and in most surgical departments the diagnosis of intestinal ischaemia relies in clinical symptoms and examination [2]. Improvement of gastrointestinal perfusion has failed to prevent mucosal failure and might affect the tissue metabolic rate [3].

Monitoring the gut metabolic markers provides information of the variation from aerobic to anaerobic metabolism characteristic of ischaemia. Measuring glucose and lactate tissue levels reveals the balance between oxygen supply and demand and therefore the ischaemic degree of that tissue.

Microdialysis, an extracting technique based on the passive diffusion of molecules against concentration gradient through an implanted hollow probe, has been used in last few decades to recover biomarkers of a broad range of tissues such as brain [4], [5], liver [6], heart [7], muscle [8] and bowel [9].

Intestinal ischaemia had been previously studied using microdialysis in experimental models [10], [11], [12]. Bowel microdialysis in humans have been performed placing the probe in the peritoneal cavity [10], [13], [14] since is less invasive than tissue implantation. However, ischaemic region changes are diluted by the metabolites from the non-ischaemic region and from the peritoneum supplied by the systemic circulation [9].

'Traditional' microdialysis studies use the commercially available analyzer from CMA (CMA 600, CMA/Microdialysis, Stockholm, Sweden). The dialysate samples are collected in vials of 200 μl volume in period ranging from 30 minutes to several hours, which are then manually stored in the freezer or in ice, by generally the ward nurse. The time burden for clinical staff and the problem of misplaced samples are serious pitfalls of this technique [5].

On-line rapid sampling microdialysis (rsMD) is characterized by a rapid detection (every 30 seconds) of the dialysate, which is directed from the microdialysis probe outlet to a biosensor assay system for analysis. The system provides a method that needs no extensive manipulation, running by itself during 24 hours up to 5 days. This biosensor assay system was previously employed in monitoring human brain trauma during and after the operation in Intensive Care Unit, measuring metabolic changes during brain ischemia [15], [16].

In order to use the on-line rsMD system to monitor human bowel for an early diagnostic of ischemia, adaptation of the on-line assay were necessary.

The current response of an enzymatic biosensor is often represented by Michaelis-Menten equation.

$$I = \frac{I_{MAX}[S]}{K_M + [S]} \tag{1}$$

Equation 1 defines the linear character of the biosensor response at low concentrations of substrate and the rapid and proportional increase of current with the increase of substrate concentration. This proportional increase in current matches to the disposition of the enzyme to be more reduced with more concentration of substrate. However, when substrate concentration becomes higher than Michaelis-Menten constant (KM), the enzyme saturates. The KM marks the limit of that linear relationship. The current becomes constant, being limited by enzyme loading and the rate of the enzyme.

In general, to reduce analytical errors in a biosensor signal, the linear range from the calibration curve is used. However, this implies a low sensitivity range available for the measurement. Mathematical expressions for biosensors calibration at different concentrations reduce the errors and quantify the length of the linear range. The Hill equation is widely used for approximations of the calibration curve in various biosensors [17].

In a FIA biosensor system such as described here, the empirical Hill equation can be used to fit the calibration curves of glucose and lactate biosensors [18],

$$I = \frac{I_{MAX}}{\left[1 + \frac{K_M}{[glucose]^{\alpha}}\right]} \tag{2}$$

where I is the measure of the rate of the enzymatic reaction and α is the measure of deviation from ideal Michaelis-Menten behaviour.

Studies to increase the sensitivity of the assay are still necessaries, such as the obtaining a wider calibration curve to meet the expected higher concentration ranges of intestinal tissue [19]. The study of the effect of enzyme concentration is an important variable. Since each enzyme contains a specific protein that recognizes a specific substrate [20], the increase of enzyme concentration can lead to an increase in enzyme sensitivity.

2.0 EXPERIMENTAL

2.1 Biosensor Assay System

The rsMD online assay has been described previously [4], [16]. Briefly, is the coupling of the microdialysis sampling technique to a flow injection analysis system (FIA). This is itself coupled to an enzyme based amperometric detector. The rapid sampling monitoring system analysed electrochemically the dialysate glucose and lactate at high time resolution (typically 30 seconds sampling). The two traces of current peaks produced are proportional in height to the concentration of substrates detected.

The rsMD data were collected at 100 Hz using a Powerlab 8/SP analogue/digital converter and Chart® 5.6 software (ADInstruments, Oxfordshire, UK) running on a Powerbook portable computer (Apple, CA, USA).

The online assay and the data collection system were placed on a clinically certified trolley (Series 7000, CTL Medical, Essex, UK) that can be transport from the laboratory to the operation theater and to the intensive care unit.

2.2 Sensitivity Effect of Enzyme Concentration

The enzymatic reactors contain two mixed cellulose esters membrane (0.025 μm pore, 25 mm, Millipore UK) placed in the direction of the flow, first the substrate oxidize and then the peroxides as described by Jones et al. [4].

The enzymes used, Glucose Oxidase (GOx: Aspergillus Niger, GLOX-70-6451, 180 U/mg protein), Lactate Oxidase (LOx: Aerococcus viridans, RELO-70-1381, 20-60 U/mg protein) and Horseradish peroxidase (HRP: Horseradish, PERO-70-6915, 180 U/mg protein) were purchased from Genzyme General Diagnostics (Genzyme Ltd.,Kent, UK).

Enzymes are adsorbed onto each membrane due to intensive filtering. Enzyme aliquots were pumped several times through a 25 mm filter holder (Swinnex, Millipore UK) where the mixed cellulose esters membrane was placed. To study the interaction of the enzymes and its behaviour at different concentrations, the membranes were loaded with (SOx: 1, 2 and 5 mg and HRP: 0.5 and 1 mg) enzymes aliquotes.

The membranes were positioned in the reactors and calibration was performed with a range of standards of D-(+)-glucose (0.5 M, Sigma, Aristar grade) and L-(+)-Lactic acid (0.5 M, Sigma, Aristar grade) from 250 μM to 30 mM. Each standard was injected three times and a current-concentration calibration curve was plotted with the three points averaged as mean ± stdev (standard deviation).

2.3 Microdialysis Monitoring of Intestinal Tissue Concentrations

Microdialysis monitoring of tissue from a colon resection surgical procedure was carried out, as a pilot study, to establish the required levels of glucose and lactate. The tunneling of the microdialysis probe in the tissue was carried out in the laboratory 30 minutes after resection and this was monitored for approximately 2 hours.

The microdialysis probe inlet tubing was connected to a CMA 400 microdialysis syringe pump (CMA/Microdialysis, Stockholm, Sweden). A saline solution (perfusion fluid: 147 mM sodium, 4 mM potassium, 2.3 mM calcium, 156 mM chloride) was perfused at 2 μl/min using the microdialysis pump. The outlet of the probe was adapted to connect to a dual online assay system [16]. Typically, a sterilized 1-meter long of low volume connection tubing (Microbiotech, Stockholm, Sweden) was used between the tissue and the online assay to reproduce the same scenario as with patients [15].

2.4 Data Analysis

The rsMD data was analyzed using Chart 5.6. Typically the glucose and lactate concentration traces were plotted over time for further analysis. When necessary more intense processing techniques were apply to remove noise using Matlab 7.5 (MathWorks, Natick, MA, USA) [21]. The delay of 9 minutes due to the 1-metres length low volume tubing connection between the tissue and the assay was considered in order to align the dialysate concentration series to real time.

Transient changes in opposite direction are recognized as physiological events. While transient parallel changes cannot be differentiated from their physiological or non physiological nature. The later can be due to the performance of the assay or the microdialysis recovery and are typically excluded of analysis [5]. Graphs and analysis were performed with Igor Pro 6 (WaveMetrics, Portland, OR, USA).

3.0 RESULTS AND DISCUSSION

3.1 Sensitivity Effect of Enzyme Concentration

The response of the enzymatic reactor when loaded with different enzyme quantities is observed in Figure 1 for GOx:HRP pair and Figure 2 for LOx:HRP pair as the average of the three injected standards (mean ± stdev).

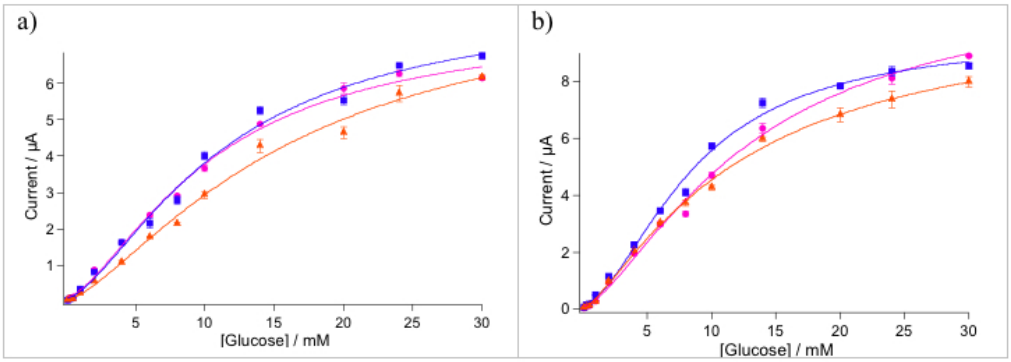

Figure 1 Calibration test varying GOx and HRP loaded in the membrane

Results from FIA system with enzyme reactor membranes loaded with 1 ml of solution containing different amount of GOx (cicle: 1 mg, square: 2 mg, triangle: 5 mg) on first membrane and HRP on second membrane. a) 0.5 mg of HRP - Hill equation fitting curves, respectively: $I = (7.69 \pm 0.62) / [1 + (10.45 \pm 1.32 / x)^{1.53 \pm 0.20}]$; $I = (8.26 \pm 0.91) / [1 + (11.26 \pm 1.91 / x)^{1.53 \pm 0.25}]$; $I = (8.65 \pm 1.4) / [1 + (16.13 \pm 4.01 / x)^{1.43 \pm 0.23}]$; b) 1 mg of HRP - Hill equation fitting curves, respectively: $I = (11.57 \pm 0.18) / [1 + (12.99 \pm 1.92 / x)^{1.47 \pm 0.18}]$; $I = (9.62 \pm 0.52) / [1 + (8.52 \pm 0.69 / x)^{1.76 \pm 0.21}]$; $I = (10.45 \pm 0.76) / [1 + (12.23 \pm 1.51 / x)^{1.31 \pm 0.12}]$

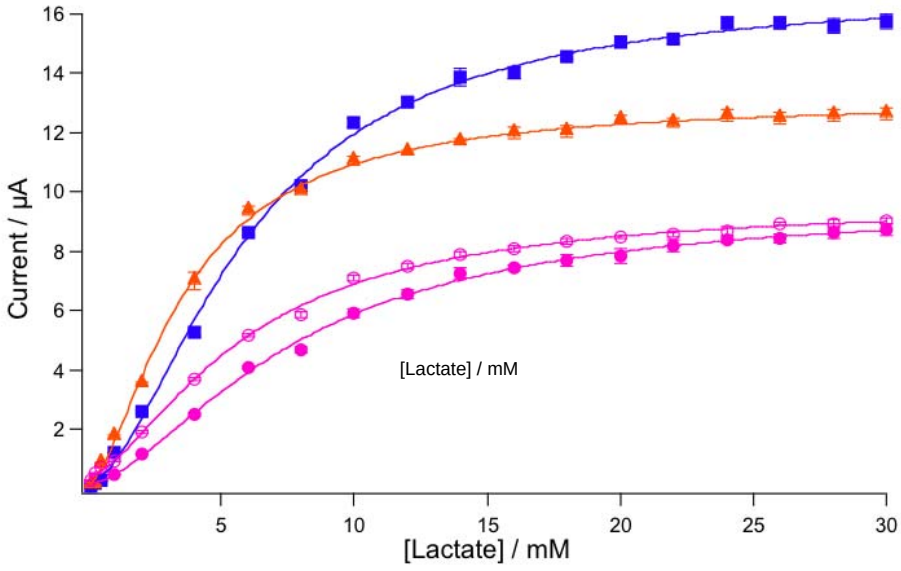

Figure 2 Calibration test varying LOx loaded in the membrane

Results from FIA system with enzyme reactor membranes loaded with 1 ml of solution containing different amount of LOx (circle: 1 mg, square: 2 mg, triangle: 5 mg), on first membrane and 0.5 mg HRP on second membrane. The Hill equation fitting curves, respectively are $I = (9.65 \pm 0.19) / [1 + (7.76 \pm 0.25 / x)^{1.63 \pm 0.08}]$; $I = (16.84 \pm 0.26) / [1 + (6.01 \pm 0.17 / x)^{1.73 \pm 0.09}]$; $I = (13.08 \pm 0.11) / [1 + (3.63 \pm 0.09 / x)^{1.59 \pm 0.06}]$. And open circles 1:1 for LOx:HRP. $I = (9.64 \pm 0.14) / [1 + (5.82 \pm 0.016 / x)^{1.58 \pm 0.07}]$

The enzymes immobilised on the membrane presented different KM and IMAX depending on the ratio SOx: HRP (Table 1)

Table 1 Experimental kinetic constants for the reactor GOx:HRP and LOx:HRP

GOx:HRP	**K_M, mM**	**I_{MAX}, µA**
1:0.5	10.45 ± 1.32	7.69 ± 0.62
2:0.5	11.26 ± 1.91	8.26 ± 0.91
5:0.5	16.13 ± 4.01	8.65 ± 1.4
1:1	12.99 ± 1.92	11.57 ± 0.18
2:1	8.52 ± 0.69	9.62 ± 0.52
5:1	12.23 ± 1.51	10.45 ± 0.76
LOx:HRP	**K_M, mM**	**I_{MAX}, µA**
1:0.5	7.76 ± 0.25	9.65 ± 0.19
2:0.5	6.01 ± 0.17	16.84 ± 0.26
5:0.5	3.63 ± 0.09	13.08 ± 0.11
1:1	5.82 ± 0.02	9.64 ± 0.14

The difference in response for different GOx and LOx loading were negligible in both HRP loading cases. Further comparison between responses can be observed in Figure 3, where the data points for 10 mM glucose and lactate are illustrated.

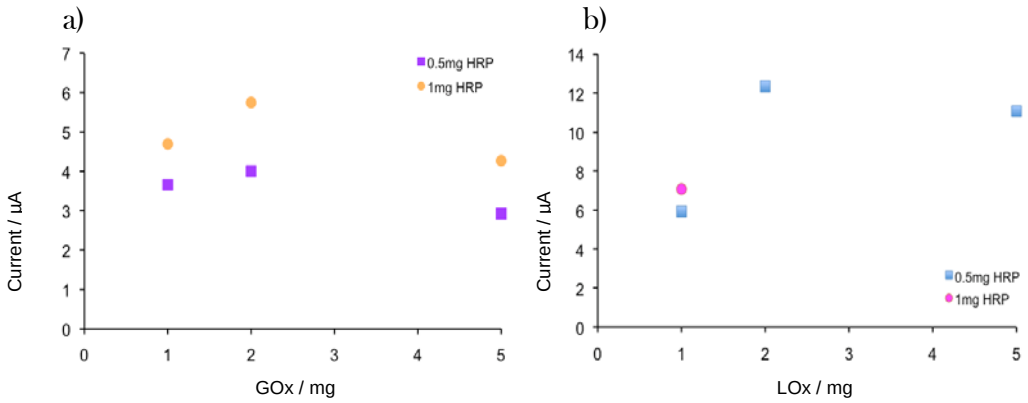

Figure 3 Response of membranes loaded with different GOx:HRP and LOx:HRP ratio

Data from Figure 1 & 2 at a) 10 mM of glucose and b) 10 mM of lactate. Response current from enzyme reactor membranes loaded with 1 ml of solution containing 1, 2 and 5 mg of SOx. 0.5 mg of HRP spuares and 1 mg of HRP circles (stdev typically smaller than markers)

The amount of GOx and LOx loaded on the membrane, increased from 1 mg to 2 mg and 5 mg did not present a significant increase in sensitivity. This confirms that the detection method is limited by mass transport and not from the kinetics of the enzymes. This mass transport limited rate provides some advantages; the possibility of depletion of oxygen in the buffer is essentially eliminated and enzyme losses through constant use do not affect significantly to the reactor sensitivity. In the case of Glucose Oxidase, the optimum membranes' loading was concluded to be 1 mg of GOx and 0.5 mg of HRP. For Lactate Oxidase, however, the increase in sensitivity when 2mg of LOx was loaded on the membrane compared with 1 mg resulted in an optimal loading for the lactate reactor of an enzyme ratio of 2:0.5 for LOx:HRP

3.2 Extended Concentration Calibration of the System

For the brain use, the on-line system was routinely calibrated between 50 µM and 2 mM [16]. For the gastrointestinal research much greater concentrations were expected [19], therefore an extended calibration range was examined.

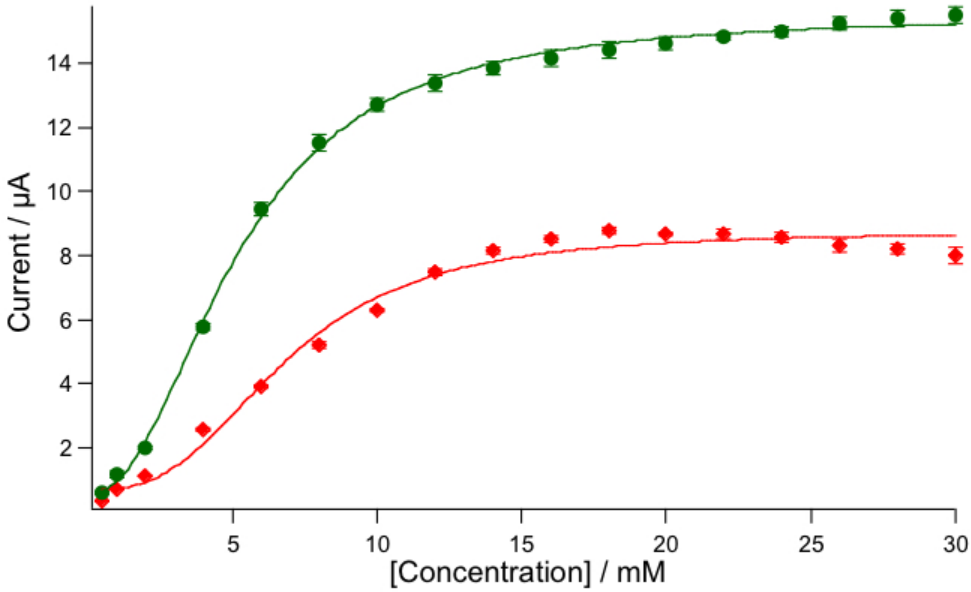

Figure 4 Extended calibration curve for Glucose and Lactate

Glucose (diamonds curve) and Lactate in (circles curve) show a pseudo-linear response until approximately 15 mM and 10 mM respectively, where the enzymes saturate and the response plateu. Samples were 200 nl injection and are represented by mean ± stdev. Hill equation fitting curves:

$$I = (8.76 \pm 0.23) / [1 + (6.87 \pm 0.37 / x)^{2.84 \pm 0.42}];$$
$$I = (15.54 \pm 0.13) / [1 + (5.18 \pm 0.11 / x)^{2.19 \pm 0.09}]$$

The KM of Glucose Oxidase and Lactate Oxidase are known to be 33 mM [22], 0.94 mM [23] respectively, marking the limit of that linear relationship. Hence, while the mode of operation for glucose (Km of GOx in a biosensor was 7.01 mM [24]) is consistent with this being a limiting factor it seems that with lactate other features acquired importance. The KM of an enzyme represents the strength of the binding protein to the substrate. The lower the KM, the stronger is the bond of the enzyme with the substrate, thus being the limiting step of the kinetic reaction. Georganopoulou et al. found in early version of the system that the response was limited above 0.2 mM of glucose concentration by HRP enzyme kinetics and enzyme loading [18]

Table 2 Comparison of K_M values from the literature and experimentally

Enzyme	K_M literature, mM	K_M experimental, mM
GOx	33 [22]; 41.8 [25]	6.87 ± 0.37
LOx	0.28 [26]; 0.94 [23]	5.18 ± 0.11
HRP	0.0334 [27]	---

The lower KM of the LOx enzyme was overcome by reducing to 200 nl the volume of the injecting sample, increasing to 2 mg the loading of enzyme in the membrane and using higher flow rates to lower the recovery by the microdialysis probe. The saturation of LOx was evaded in this way and the enzyme was never deficient of active sites to oxidase the substrate.

The lower sensitivity of glucose observed in Figure 4 is due to the nature of this sugar. Glucose is a mixture of 2 anomers α-Glucose (40%) and β-Glucose (60%), that are interconverted at low rates in solution. Glucose Oxidase is specifically selective to the β-anomer, then it is β-(+)-glucose that reacts actively with the enzyme, generating a 60% of the total response.

3.3 Microdialysis Monitoring of Intestinal Tissue Concentration

The superior dialysate concentrations range for glucose and lactate expected in intestinal tissue were confirmed with the pilot study carried out in a tissue sample from a colon resection surgical procedure.

Figure 5 shows the decrease of Glucose concentration from 300 - 400 μM to 15 μM and the increase of Lactate from 40 μM to approximately 6 mM, having a sharp transition half an hour after the beginning of the monitoring. This rise in lactate levels and drop in glucose levels represent the metabolic changed when a healthy bowel tissue reaches ischaemic conditions.

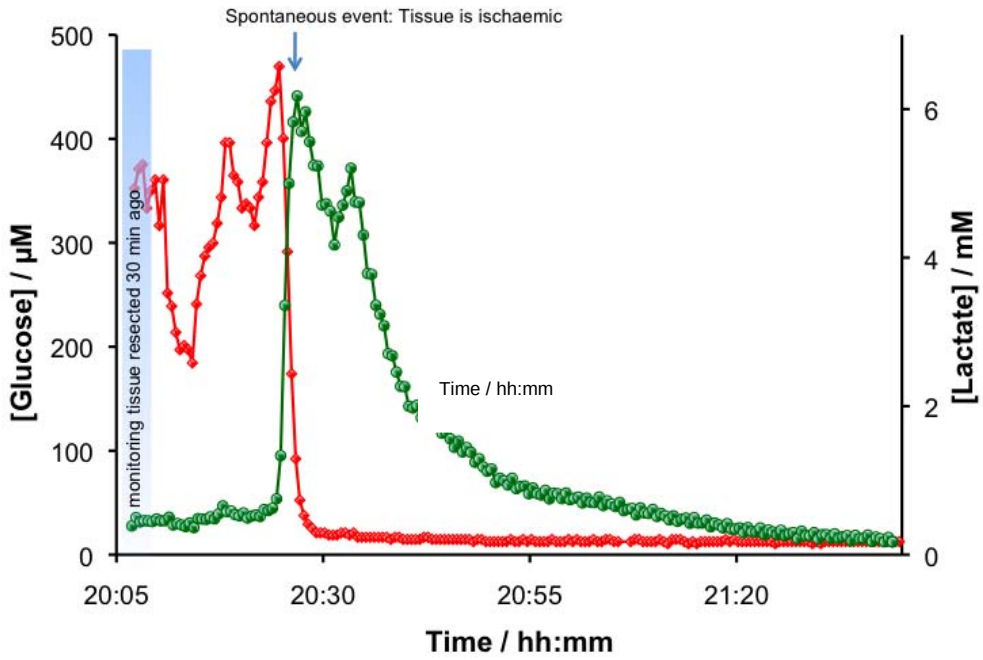

Figure 5 Human resected tissue monitoring

Concentration changes of glucose (diamonds) and lactate (circles) in a cadaveric tissue. Probe is implanted in a section of bowel resected during an operation 30 minutes before. Metabolites levels are constant during the first half an hour to then change in opposite directions spontaneously indicating that the tissue is ischaemic

These results establish the detection limits of the biosensor reactors. Since the tissue was monitored 1 hour after resection it can be considered that ischaemia had already set in [13]. We might conclude for a calibration method with standards from 250 µM to 6 mM. The limiting factor of the theoretical KM for LOx was overcome with this new variant of the on-line rapid sampling microdialysis system. Therefore, for gastrointestinal monitoring, wider concentration range could be monitored unlike the lower series of brain microdialysis that range between 50 µM and 2 mM.

The adaptation of the system was further developed in the in vivo monitoring, in the theater. The tunneling of the probe into the tissue was modified in every performance. The data obtained confirmed the used of the system for on-line monitoring of metabolic changes in gastrointestinal tissue and it was validated during bowel surgeries [28].

5.0 CONCLUSION

On-line rapid sampling microdialysis to monitor ischaemia durign bowel surgery was succesfully adapted from the previously assay used to study biochemicals in traumatic brain injury.

Unvariability of the response while enzyme concentration was increased confirmed the mass transport limited rate of the biosensor detection. Lost of sensitivity due to oxygen depletion or enzyme losses were eliminated as the limiting factor was confirmed not to be the enzymes kinetics.

Sensitivity increase was achieved reducing the volume of the injecting sample, increasing the loading of enzyme in the membrane and reducing microdialysis probe recovery efficiency by increasing the flow rates of the perfusate compared with the previous assay used.

Although the adaptation of the system to monitor intestinal ischaemia was genuinely validated in vivo during colon resections procedures. The pilot run, monitoring cadaveric tissue, confirmed the higher concentration range expected in healthy and ischemic tissue.

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