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Quantification of pyruvate in-vitro using mid-infrared spectroscopy: Developing a system for microdialysis monitoring in traumatic brain injury patients

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ABSTRACT

Introduction: Complex metabolic disruption is a major aspect of the pathophysiology of traumatic brain injury (TBI). Pyruvate is an intermediate in glucose metabolism and considered one of the most clinically informative metabolites during neurocritical care of TBI patients, especially in deducing the lactate/pyruvate ratio (LPR) – a widely-used metric for probing the brain's metabolic redox state. LPR is conventionally measured offline on a bedside analyzer, on hourly accumulations of brain microdialysate. However, there is increasing interest within the field to quantify microdialysate pyruvate and LPR continuously in near-real-time within its pathophysiological range. We have previously measured pure standard pyruvate in-vitro using mid-infrared transmission, employing a commercially available external cavity-quantum cascade laser (EC-QCL) and a microfluidic flow cell and reported a limit of detection (LOD) of 0.1 mM.
Research question: The present study was to test whether the current commercially available state-of-the-art mid-infrared transmission system, can detect pyruvate levels lower than previously reported.
Materials and methods: We measured pyruvate in perfusion fluid on the mid-infrared transmission system also equipped with an EC-QCL and microfluidic flow cells, tested at three pathlengths.
Results: We characterised the system to extract its relevant figures-of-merit and report the LOD of 0.07 mM.
Discussion and conclusion: The reported LOD of 0.07 mM represents a clinically recognised threshold and is the lowest value reported in the field for a sensor that can be coupled to microdialysis. While work is ongoing for a definitive evaluation of the system to measuring pyruvate, these preliminary results set a good benchmark and reference against which future developments can be examined.

1. Introduction

Traumatic brain injury (TBI) is an important worldwide public health issue and a major cause of death and disability (Maas et al., 2015). After the mechanical impact (primary injury), deleterious mechanisms such as brain metabolic dysfunction trigger the propagation of a secondary brain injury (Venturini et al., 2023). Despite great efforts in management of secondary TBI during neuro-critical care, mortality remains high and third of the survivors have poor neurological and functional outcome (Bramlett and Dietrich, 2015).

The management of severe TBI patients in neurocritical care involves monitoring of various parameters in a multi-modal approach. These parameters may include intracranial pressure, brain tissue oxygenation and brain metabolism (Le Roux et al., 2014; Hutchinson et al., 2015; Rohlwink et al., 2012). The latter uses microdialysis to monitor brain metabolites including glucose, lactate, and pyruvate. The cerebral microdialysis principle and its relevance in TBI has been widely reported (Hutchinson et al., 2000; Tisdall and Smith, 2006; Helmy et al., 2009; Rostami and Bellander, 2011; Gowers et al., 2015). The lactate/pyruvate ratio (LPR) is a well-accepted metric with thresholds $\geq$25 or $\geq$40 often used as markers for ischaemia, metabolic crisis, or mitochondrial dysfunction, associated with poor prognosis (Timofeev et al., 2011;

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Guilfoyle et al., 2021). Thus, continuous assessments of extracellular brain metabolites are crucial to better understand the pathophysiology of TBI while also enabling clinicians make informed decisions. Timofeev and colleagues found that pyruvate was a negative predictor of mortality (relatively high pyruvate was associated with reduced mortality) in a logistic regression model (Timofeev et al., 2011). Nordström and colleagues have defined "ischaemia" as LPR >30 with pyruvate <70 μmol/L, while "mitochondrial dysfunction" was defined as LPR >30 with pyruvate ≥70 μmol/L, in severe TBI patients (Nordström et al., 2016). "Ischaemia" is a shortage of blood supply to an organ or tissue, meaning (in the present context) that the brain, or part of the brain, does not receive enough oxygen and nutrients for normal function. "Mitochondrial dysfunction" is a broad term that can encompass imbalances or defects in production of high-energy phosphates by mitochondria, which may stem from problems with the Krebs cycle and/or mitochondrial electron transport chains. "Metabolic crisis" is less clearly defined but is a severe state characterised by low interstitial glucose and high LPR (Nordström et al., 2016). In neurocritical care, these malfunctions are reflected in microdialysis measures.

Previously our group has reported the "first generation" mid-infrared spectroscopic system integrated with cerebral microdialysis to meet the pressing need for continuous online metabolite monitoring (Alimagham et al., 2021). The system utilises an external cavity-quantum cascade laser (EC-QCL) mid-infrared spectroscopic sensor, which offers several properties surpassing conventional spectroscopic techniques such as the Fourier-transform infrared spectroscopy (FT-IR). Unlike FT-IR thermal source which emits photons over a broad spectral range, QCLs emit their photons at defined wavelengths. This means that the spectral density of QCLs and subsequently, the signal to noise ratio is orders of magnitude higher compared to that of a thermal source (Childs et al., 2015; Yao et al., 2012). The limit of detection (LOD), defined as the lowest detectable concentration for pure pyruvate by the system was reported as 0.1 mM (Alimagham et al., 2021), using a commercially available ChemDetect mid-infrared analyzer (DRS Daylight Solutions, Inc, San Diego, CA, USA). Here, the LOD was estimated as three times the ratio of the standard deviation of the background noise to the slope of the calibration curve. The slope of the calibration curve represents the relationship between the signal response and the concentration of pure pyruvate. The equation for LOD has been presented in the Methods section.

Elsewhere, levels of pyruvate in TBI patients measured hourly by conventional offline enzymatic-colorimetric microdialysis analysers (CMA600 and ISCUSflex) have been reported as almost always <0.2 mM, and in some cases <0.1 mM (Timofeev et al., 2011; Guilfoyle et al., 2021).

Advances to optimise the mid-infrared system, in particular its sensitivity to pyruvate, are in progress. Our review of microdialysis-integrated sensors for TBI also acknowledged the common challenge within the field in quantifying pyruvate within its pathophysiological range (Zimphango et al., 2022). Among many factors, there have been limited studies focusing on pyruvate measurements using microdialysis-integrated sensors. Thus, achieving pyruvate quantification at low levels in-vitro is an important preparation for advancing continuous monitoring of brain metabolism in vivo using microdialysis-integrated sensors –an essential step to effectively predict the onset of secondary brain damage in-vivo.

Our current work determines whether the "second-generation" mid-infrared spectroscopic sensor system for brain metabolite measurements, utilising a commercially available Culpeo mid-infrared analyzer (DRS Daylight Solutions, Inc), is superior to its forerunner. This study's aim was to demonstrate the sensor's capability to measuring pyruvate at pathophysiological ranges reported in TBI patients. To achieve this, we characterised this "second generation" mid-infrared EC-QCL system to extract its relevant figures-of-merit. We also lay out and discuss the findings, current limitations, and future directions for the study.

2. Methods

2.1. Non-technical overview of methods

- Mid-infrared (mid-IR) spectroscopy is an analytical technique that allows characterisation and quantification of molecules, by virtue of detecting vibrations within the molecules.
- We are interested in the utility of mid-IR to measure glucose, lactate, and pyruvate, which are clinically and metabolically important molecules in brain interstitial fluid, which can be sampled by a technique called microdialysis.
- Test solutions of known concentrations were prepared from commercially available sodium pyruvate and used to assess the sensitivity of the mid-IR spectrometer.
- The mid-IR spectrometer uses the most sensitive technology commercially available, the important characteristic of this is the employment of quantum cascade lasers.

2.2. Reagents and solutions

All reagents were analytical grade, purchased from Sigma-Aldrich (Poole, Dorset, UK). Standard solutions of pyruvate within the relevant clinical concentration ranges were prepared by dissolving sodium pyruvate in an in-house microdialysis perfusion fluid (147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$ in ultrapure water), which is the same composition as M Dialysis CNS perfusion fluid marketed for cerebral microdialysis.

2.3. Experimental setup for mid-infrared measurements

A commercially available QCL-IR instrument (Culpeo analyzer, DRS Daylight Solutions, Inc.) was used for acquiring laser-based infrared spectra in the range of 950–1250 cm^{-1} wavenumbers. An external water chiller was set to 20 °C and left to reach thermal equilibrium for at least 30 min before data acquisition to ensure thermal stabilisation of the laser measurements. A programmable Microfluidic Modular syringe Pump SPM -100 (MicroNano Tools/Micromolding Solutions Inc, Abbotsford, Canada) was used to pump perfusion fluid with or without pyruvate, loaded in 2.5 mL syringes. A Hamilton Modular Valve Positioner (MVP) with six defined valve modes and controller (Hamilton Central Europe, Giarmata, Romania) was used to inject the perfusion fluid as the reference, and pyruvate solution, to the QCL-IR instrument via the connecting tubing.

Fig. 1 shows a simple diagram of the QCL-IR instrument that we used for metabolite analysis. It consists of the EC-QCL with two concave spherical mirrors, a transmission flow cell module, and a TE cooled detector. It is also designed to be coupled to a fluid delivery system allowing sample delivery via the sample inlet onto the transmission flow cell and out via the sample outlet.

EC-QCL emits radiation beam (I_0) guided orthogonally by the mirror onto the transmission flow cell causing biochemical bonds in the sample vibrate. Consequently, this leads to specific amounts of energy from the I_0 to be absorbed. This reduces the intensity of the radiation guided (I), which is again orthogonally guided by the second mirror to reach the detector. In such a configuration, Beer-Lambert's law is applied to describe the relationship between the absorbance of light by a sample, the concentration of the absorbing species in the sample, and the path length of light through the sample (Equation (1)).

$$a = \varepsilon c l \tag{1}$$

Napierian absorbance (a) is proportional to the concentration (c) and the pathlength (l) of pyruvate, as well as the molar absorptivity (ε) at a given wavelength.

For measurements, three microfluidic flow cells of 76, 100, and 120 μm pathlengths were tested. The flow rate at which pyruvate was delivered to the microfluidic flow cell was 50 μL/min. Spectral scans of

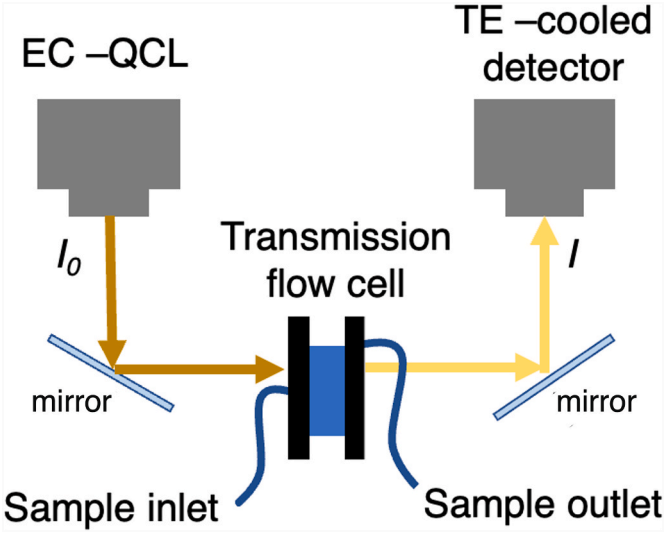

Fig. 1. Simplified schematic of a QCL-based mid-infrared transmission setup for quantification of pyruvate. Adapted from (Alimagham et al., 2021), published Open Access CC BY, copyright The Authors.

100, 200, 400, 600, 800 and 1000 averages per spectrum were tested. The in-house perfusion fluid was used as reference before each measurement to set the baseline (i.e., logarithmically converted absorbance spectrum was zero). Each sample was measured three times at ambient room temperature.

2.4. Data analysis

Signal to noise ratio (SNR) calculations were derived by using the equation below,

$$SNR = \frac{H}{h} \tag{2}$$

where (*H*) is the height of the pyruvate peak (at 1176 cm $^{-1}$) measured to a baseline, and (*h*) is the difference between the largest and smallest noise value between 1000 and 1100 cm $^{-1}$.

MATLAB (R2023a, MathWorks, Inc., Natick, MA, USA) and MS Excel were used to plot the spectra and calibration curves. Pyruvate baseline correction was performed by subtracting absorbance value at 1080 cm $^{-1}$ wavenumber from all wavenumbers across the spectrum. The LOD for pyruvate was derived using equation (3) – also previously adopted by our group to deduce LODs of pure lactate and glucose (Alimagham et al., 2021).

$$LOD = \frac{3\ x\ (noise)}{Slope\ of\ the\ calibration\ function} \tag{3}$$

3. Results and discussion

We aim to accurately characterise the novel "second-generation" mid-infrared EC-QCL spectroscopic sensor and extract its relevant figures-of-merit for the different parameters (e.g., pathlength and number of scan averages) and demonstrate its capability to measure pyruvate –a key metabolite in TBI.

3.1. Desirable parameters for optimising metabolite measurements

To maximise the quantification of pure pyruvate within clinical ranges, we deduced the desirable pathlength and number of spectral scan averages. Mid-infrared transmission spectra of 1.5 mM pyruvate in perfusion fluid were recorded for three flow cells of 76, 100, and 125 μm pathlengths at a series of spectral scan averages (100, 200, 400, 600, 800 and 1000 scans) (Fig. 2A). The prominent peak in pyruvate's spectra at 1176 cm^{-1} in Fig. 2A corresponds to the C–CH_3 stretching vibration (Kakihana and Okamoto, 1984). The time durations taken for the chosen scan averages are presented in Table 1.

The pathlength of a flow cell is defined as the distance the light travels through the sample as it passes through the cell. This is important because pathlength affects the intensity of the transmitted or absorbed light and hence sensitivity of the measurement. Here, it is apparent that the flow cell with the 76 μm pathlength yields a smoother spectrum and is less noisy in comparison to the 100 and 125 μm pathlengths respectively (Fig. 2A). This was also confirmed by calculating the SNR across the chosen scan averages for all three flow cells (Fig. 2B). All three flow cells show a pattern whereby the SNR reaches a plateau with an increasing number of scan averages. The 76 μm flow cell clearly shows the best (largest) SNR and reaches a plateau such that 400 scan averages is an optimal acquisition period – no further gain in SNR was achieved by 600–1000 scan averages.

These findings utilising the Culpeo mid-infrared analyzer (DRS Daylight Solutions, Inc.) agree with our previous work, where the 76 μm pathlength was optimal for similar measurements using the previously reported mid-infrared sensor (ChemDetect analyzer, DRS Daylight Solutions, Inc.) (Alimagham et al., 2021).

The importance of scan averages in spectroscopy lies in their ability to reduce the effects of random noise and improve the SNR of the spectral data. The optimal number of scan averages depends on several factors, including the nature of the sample, sensitivity of the

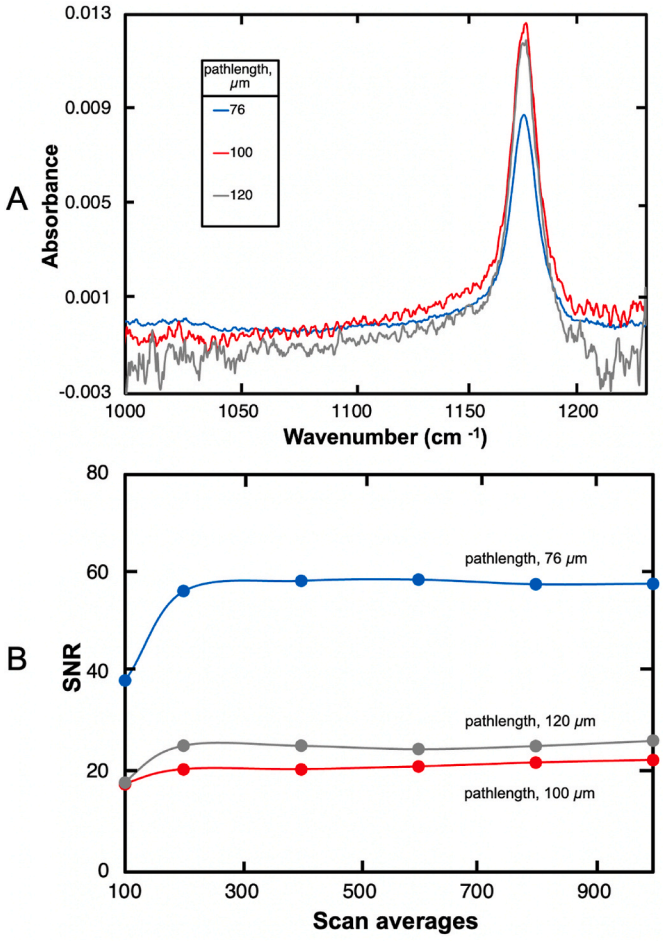

Fig. 2. Evaluation of the mid-infrared sensor's performance. (A) Mid-infrared spectra of 1.5 mM pyruvate in flow cells with pathlengths of 76, 100, and 125 μm. (B) The data were then used to deduce relative noise levels (signal-to noise ratios, SNR) versus the various numbers of spectral scans averaged.

Table 1
Scan averages and acquisition times for measuring pyruvate concentrations at clinically relevant levels.

Scan averages	100	200	400	600	800	1000
Time/s	50	100	200	300	400	500

instrumentation, as well as the desired level of precision and accuracy. Thus, increasing the number of scan averages improves the SNR of the measurement. However, after a certain point, there are diminishing returns as the plot of SNR can effectively reach a plateau with increasing number of scans. We found that 400 scan averages with 200 s acquisition time to be optimal for obtaining the best spectral quality data within a reasonable time frame (Fig. 2B). Though this was determined using pure pyruvate, 400 scans would also be applicable when measuring other metabolites or microdialysates.

3.2. Established limit of detection within pathophysiological range in TBI

We estimated the LOD indirectly through a linear calibration function constructed from a linear regression. This was achievable by calibrating the performance of the system through a concentration series of pyruvate in perfusion fluid (Fig. 3A). Through this simple model, additional information about accuracy, precision, quantification limit, and linearity were calculated. Following the Beer-Lambert's law (Equation (1)), we obtained a linear calibration line (Fig. 3B) through which, we were able to determine the LOD of pyruvate as 0.07 mM. The exact concentrations of extracellular pyruvate after TBI depend on several factors, including severity and stage of the injury. Involvement of pyruvate in TBI and the significance of the LOD reported here is discussed in the subsequent text below.

The LOD of 0.07 mM for pyruvate that we determined here, using Equation (3) above, for the Culpeo is better (lower) than that which we determined for the previous model of mid-IR detector, the ChemDetect (DRS Daylight Solutions, Inc), which gave an LOD of 0.1 mM for pyruvate using the same equation (Alimagham et al., 2021). For the Culpeo, it can be seen in Fig. 3B (inset) that 0.05 mM and 0.06 mM pyruvate gave discernible absorbances greater than for 0 mM pyruvate, but using Equation (3), which considers noise, the LOD emerges as 0.07 mM pyruvate.

To set these limits in context, the conventional clinical microdialysis bedside analyzer – the ISCUSflex (M Dialysis AB, Stockholm, Sweden), like its predecessor the CMA600 - has a reported "detection limit" of 0.01 mM for pyruvate, according to the manufacturer's technical

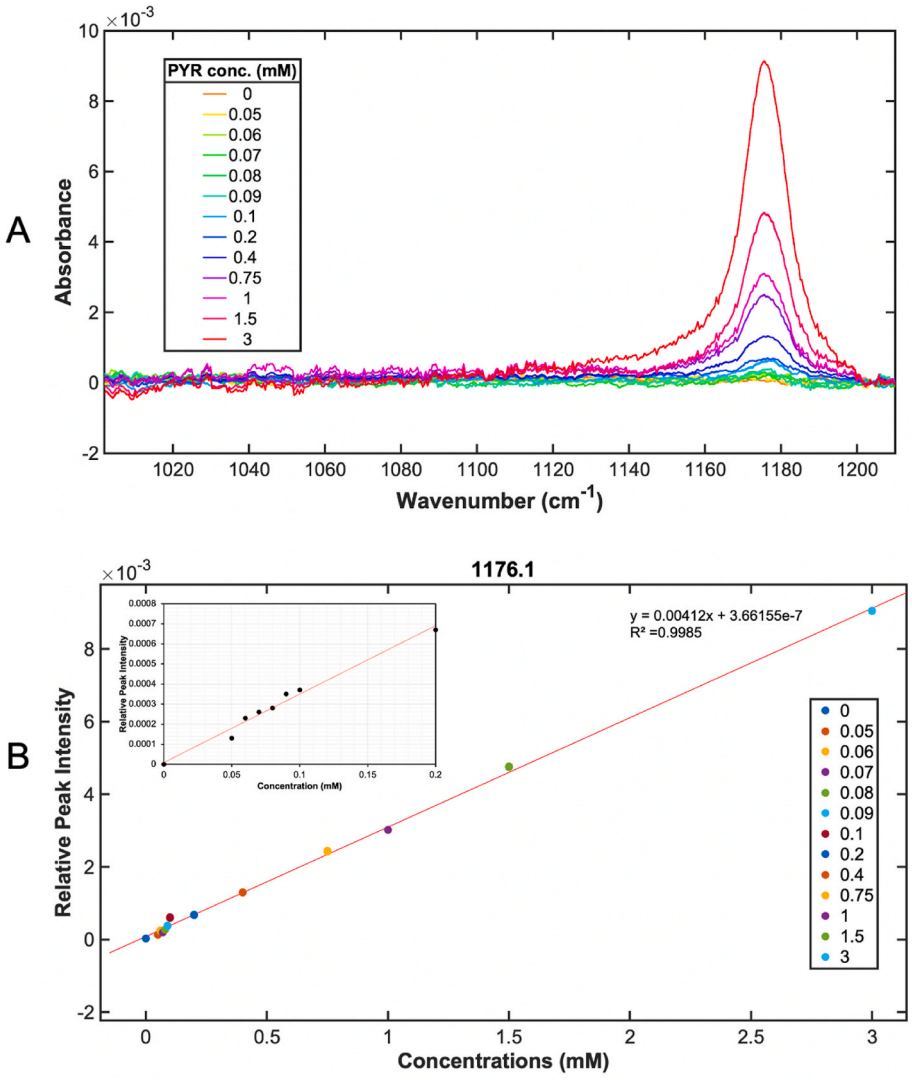

Fig. 3. (A) Absorbance spectra of pyruvate at clinically relevant levels measured on the spectroscopic sensor and (B) its calibration curve at 1176.1 cm^{-1} for three repeats. Absorbance values were baseline corrected as previously explained in the Methods section. The equation of the fitted line is y = 0.0412x + 3.66155e-7 (r^2 = 0.9985). The error bars are invisible because they are smaller than the size of the datapoints. The inset shows data points below 0.2 mM.

manual. This was determined using known concentrations of pyruvate, although the manufacturer did not define how that detection limit was calculated. The ISCUSflex employs enzymatic-colorimetric detection, done off-line with manual hourly transfer of microdialysate collection vials. In contrast, our mid-IR system if implemented in a clinical setting would be able to take measurements in a near-real-time continuous manner without necessitating manual transfers.

To set our interest in pyruvate in its biochemical context, pyruvate is a key metabolite involved in glucose metabolism and considered one of the most clinically informative in neurocritical care, along with glucose and lactate (Hutchinson et al., 2015). Pyruvate is derived from glucose after entering the glycolytic pathway generating two molecules of ATP per molecule of glucose. In the presence of oxygen, pyruvate enters the TCA cycle yielding further ATP molecules. The yield per molecule of glucose metabolized fully to CO_2 by glycolysis followed by mitochondrial respiration is theoretically 36–38 molecules of ATP, although the actual yield is believed lower (Lodish et al., 2000; Berg et al., 2019). The number of ATP molecules generated per mole of glucose is lower under conditions of hypoxia or mitochondrial dysfunction, as glycolysis only generates 2 molecules of ATP per molecule of glucose. Also, when glucose levels are low, the production of ATP will be consequentially low. Brain tissue cells attempt to compensate for diminished ATP production by upscaling glucose metabolism via glycolysis, which does not require molecular oxygen. Pyruvate is converted to lactate by the action of the enzyme lactate dehydrogenase during which NADH is recycled to NAD^+, facilitating the maintenance of glycolysis. This process leads to a rise in lactate and the LPR, and although pyruvate levels were an independent predictor for mortality in a logistic regression whereby low pyruvate was associated with increased mortality (Timofeev et al., 2011), the reliable and well-used metric is the LPR (Hutchinson et al., 2015).

In a study of TBI patients, the median levels of brain microdialysate pyruvate were around 0.1 mM seven days after the injury (Timofeev et al., 2011). Guilfoyle and colleagues found mean pyruvate levels of over 0.1 mM, over a period of two weeks from the initial brain trauma (Guilfoyle et al., 2021). The LOD reported in the present study for pyruvate of 0.07 mM is a major step towards the deployment of mid-infrared spectroscopy for online detection of microdialysate pyruvate at low levels in TBI patients. Notably, 0.07 mM (=70 μmol/L) represents a clinically recognised threshold, whereby "ischaemia" is defined as LPR >30 with pyruvate <70 μmol/L (<0.07 mM), while "mitochondrial dysfunction" is defined as LPR >30 with pyruvate ≥70 μmol/L (≥0.07 mM), in severe TBI patients (Nordström et al., 2016).

3.3. Clinical application and added value

Use of mid-IR near-real-time detection will enable clinical microdialysis, currently limited to hourly measurements with conventional bedside analysers, to work more effectively in multimodality monitoring alongside continuous measurement of intracranial pressure (ICP) and brain tissue oxygen tension ($PbtO_2$). The proposed clinical setup is illustrated in Fig. 4. The three probes (microdialysis, ICP and $PbtO_2$) are inserted through the same burr-hole via an established triple lumen cranial access device. A continuous mid-IR microdialysis sensor would save nurses' time (eliminating manual vial transfers) and would respond to rapid changes missed by hourly readings. Microdialysis would then be comparable with ICP and PbO_2 allowing integration of near-continuous microdialysis monitoring with software such as ICM+, maximising efficiency by displaying all these readouts simultaneously on one screen, so that clinical staff can see at-a-glance the state of the patient's brain. Having such a fully integrated multi-modality capability will also provide enhanced opportunities for further research on the interactions between ICP, $PbtO_2$ and brain chemistry, and may lead to discovery of ways of improving treatment for the patients leading to better clinical outcomes.

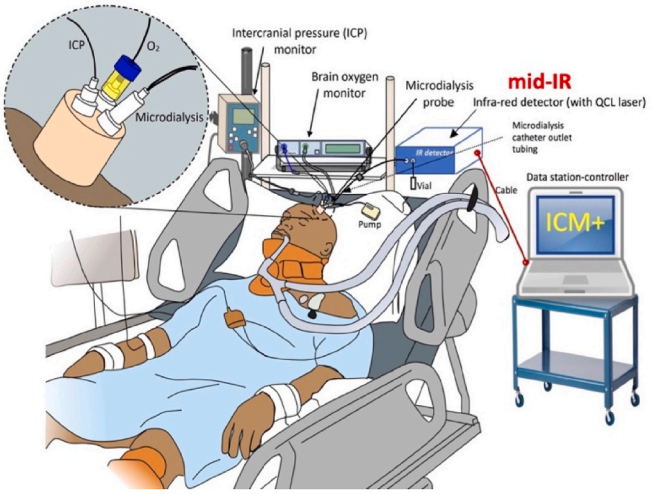

Fig. 4. Proposed clinical setup of multimodality monitoring utilising mid-IR continuous microdialysate analysis alongside intracranial pressure and brain tissue oxygen measurements. This Figure is based on an illustration copyright Susan Giorgi-Coll, adapted here with her permission.

4. Conclusion and future directions

We have demonstrated the capability of a mid-infrared spectroscopic system to quantify pyruvate down to concentrations as low as 0.07 mM, a clinically recognised threshold (see above). To our knowledge, this is the lowest pyruvate value reported for a spectroscopic sensor that can be coupled to microdialysis. This report should be regarded as preliminary, as work is ongoing for further evaluation of the system to measure pyruvate. The current results set a good benchmark and reference for assessing future developments. While future developments may enable even more sensitive detection, we regard our present achievement of 0.07 mM pyruvate as already sufficient to be clinically useful. We are directly extending this study to characterise pyruvate levels within microdialysates in-vitro. This will then be followed by offline and ultimately online measurements of patients' brain microdialysates using mid-infrared sensing to further provide direct insights of detectable brain extracellular pyruvate levels in TBI.

Authorship confirmation statement

C.Z., F.C.A., T.H., P.J.H., and K.L.H.C. contributed to the study conception and design. The data collection was performed by C.Z. Data analysis was performed by C.Z. and F.C.A. The first draft of the manuscript was written by C.Z. and all authors contributed thereafter to the subsequent versions.

Declaration of competing interest

The Authors declare the following competing financial interest: F.C.A., K.L.H.C., P.J.H., and T.H. are inventors on a patent application related to the described research in the present study and in Alimagham et al., (2021). The Authors declare no other competing financial interest.

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