INTRODUCTION

Oxidative stress occurs when there is an imbalance between formation and regulation of reactive oxygen species (ROS) in cells and tissues, causing ROS levels to accumulate (Zorov et al., 2014). This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism. Oxidative stress influences many physiological processes including the immune system and cellular communication (Reuter et al., 2010), and has been linked to intense exercise, inadequate diet, ageing and several age-related disorders, as well as many chronic diseases (Liguori et al., 2018; Lobo et al., 2010; Simioni et al., 2018). Some of the chronic diseases shown to be associated with increased levels of oxidative stress include cardiovascular diseases, including vascular diseases, high cholesterol, stroke, heart failure and hypertension (Madamanchi et al., 2005); cancer (Sosa et al., 2013); Parkinson’s disease (Wei et al., 2018); Alzheimer’s disease (Huang et al., 2016); diabetes (Asmat et al., 2016); kidney disease (Daenen et al., 2019); rheumatoid arthritis (Quiñonez-Flores et al., 2016); sepsis (Mantzaris et al., 2017); and respiratory distress syndrome (Brigham, 1990; Marseglia et al., 2019).

Protein carbonyls (aldehydes and ketones) represent a marker of overall oxygen metabolism and oxidative stress (Dalle-Donne et al., 2003; Fedorova et al., 2014; Winterbourn et al., 2000), as they are generated by multiple different reactive oxygen species in blood, tissues and cells (Frijhoff et al., 2015). Protein carbonyls are abundant in plasma, chemically stable, and an easily detectable marker of oxidative stress using a wide variety of laboratory-based analytical methods to date have required significant sample preparation and do not provide the ability to measure dynamic changes. To overcome this problem, we develop the first method for measuring in vivo concentration changes of protein carbonyls using a minimally invasive optical fibre biosensor. We use this biosensor to measure redox balance and oxidative stress within living systems by measuring dynamic in vivo concentration changes of protein carbonyls.
techniques (Weber et al., 2015). These methods for measurement of carbonyl content in biological samples were developed in the early 1970s and are still applied today. The main methods range from simple spectrophotometric analysis to liquid chromatography and mass spectrometry. All the current methods are in vitro and require careful sample handling and preparation and reported protein concentrations differ considerably depending on the applied protocols (Weber et al., 2015). For example, ELISA is considered the best available method to quantify protein carbonyl concentrations, whereas immunoblotting allows comparable detection of the molecular weight of oxidized proteins (Augustyniak et al. 2014). These methods are widely used for the interpretation of changes in redox balance due to exercise, diabetes, cellular damage, ageing and age-related disorders (Gonos et al., 2018). While these methods for measuring protein carbonylation have been implemented in different laboratories around the world, to date no methods prevail as the most accurate, reliable and robust (Rogowska-Wrzesinska et al., 2014). All of the current methods for measuring protein carbonylation require sample preparation and yield information that is static in nature and therefore unable to measure dynamic changes in protein carbonylation. It would be far more advantageous to have a small calibrated device that can continuously monitor in vivo concentrations of protein carbonyls. This would be highly compatible with optical fibres as a sensing modality since the molecular specificity in these approaches is derived from recognition molecules, such as chemosensor fluorophores that specifically target chosen analytes (Heng et al., 2013). Fluorescence is highly compatible with optical fibres as a sensing modality since the chemosensors can be attached either along the length or at the tip of the fibre surface (Kostecki et al., 2014). The chemosensors can then be excited by the light guided along the fibre, and the resulting fluorescence signal is collected via the same fibre. Optical fibre probes offer a minimally invasive approach to creating such a device for reporting in vivo physiological signals (Li et al., 2018). The key benefit of optical fibres for in vivo sensing is their small size and their inert glass composition, allowing for minimally invasive implantation, and non-destructive probing deep within tissue. They make it possible to reduce the sensing area down to hundreds or tens of microns, so that molecular information on a local scale can be obtained, and yet with a signal to noise ratio sufficient to capture specific rare and rapid molecular events (Kostecki et al., 2018). Critically, such light-based sensing technologies lend themselves to the repeated measurement of in vivo molecular events. The molecular specificity in these approaches is derived from recognition molecules, such as chemosensor fluorophores that specifically target chosen analytes (Heng et al., 2013). Fluorescence is highly compatible with optical fibres as a sensing modality since the chemosensors can be attached either along the length or at the tip of the fibre surface (Kostecki et al., 2014). The chemosensors can then be excited by the light guided along the fibre, and the resulting fluorescence signal is collected via the same fibre. Optical fibre-based biosensors have been shown to detect a wide range of biologically relevant molecules such as viruses, toxins, drugs, antibodies, tumour biomarkers and tumour cells, glucose and a wide range of biochemicals (Bosch et al., 2007). Different strategies have been used to functionalize fibre cores with chemosensors, such as covalently attaching silanes, physical attachment of charged polymers (polylelectrolytes), thin-film deposition of doped polymers, or introducing functional groups on the fibre surface with surface linking chemistry (Bachuwa et al., 2019).

Here, we present initial results of an optical fibre biosensor device, made by combining 3D printed needle technology with an optical fibre tip sensor, to produce an optical fibre-based reversible protein carbonylation biosensor capable of providing in vivo information about dynamic changes in oxidative stress levels and redox balance.

2 | EXPERIMENTAL SECTION

2.1 | Fibre tip sensor

A protein carbonyl sensor known to reversibly bind to protein carbonyls and induce fluorescence (495/517 nm Ex./Em. Max) was added to liquid acrylate polymer to create a composite material. 600 μm patch fibre (Thorlabs; M29L05) was cut in half and stripped back to expose the solid glass core, which was then cleaved. The tip of the glass core was functionalized by dipping the tip into the sensor-polymer composite material for 1 s. This viscous polymer composite left behind at the fibre tip was then polymerized to form a solid bead at the end of the fibre. Next, the tip of the fibre was dipped cleaned by quickly dipping into a solution of 50/50 ratio acetone and isopropanol (IPA) followed by quickly dipping into a solution of only IPA. This procedure left behind a solid layer of sensor-polymer composite at tens of micrometres thick on the tip of the cleaved M29L05 patch fibre to create a fibre tip oxidative stress sensor (tip sensor).

2.2 | Measurement set-up

As illustrated in Figure 1, the fully fibre coupled set-up is comprised of a 488 nm laser light source (Integrated Optics MatchBox CW) and shutter to send a 0.1 s pulse of 130 μW excitation light to the tip sensor via a bifurcated fibre bundle (Thorlabs; RP21). The back-reflected signal was coupled back into the bifurcated fibre bundle, passed through a 488 nm long-pass edge filter (Semrock; BLP01-488R-25) and characterized using an Ocean Optics QE Pro spectrometer.

![Figure 1: Schematic of the measurement set-up](Image)
2.3 | β-hydroxybutyrate (BHB) characterization solutions

A 0.5 mM BHB stock solution was prepared by reconstituting the contents of a β-hydroxybutyrate Standard vial (Cayman Chemical Company; 700192), which contains a lyophilized powder of DL-hydroxybutyrate, with 2 ml of β-Hydroxybutyrate Assay Buffer (Cayman Chemical Company; 700191). The reconstituted solution is stable for 6 h on ice. The standard curve was obtained immediately by using the set-up to measure the stock solution and then reducing concentrations of the BHB solution. These reducing concentrations were prepared by adding an additional appropriate volume of β-Hydroxybutyrate Assay Buffer to the 0.5 mM BHB stock solution to obtain a stepwise change in concentration, as shown in Table 1.

2.4 | 3D printed needles

The hollow microneedles were designed using Solid-works software, and the bore sizes of microneedles were determined based on the diameters of the fibre tip sensor and capillary. The predesigned hollow microneedles were produced from Class-IIa biocompatible e-shell 300 acrylate-based photopolymer (Envision TEC) using an Envisiontec Perfactory stereolithography machine. The needle design is shown in Figure 2(a); the final 3D printed microneedle is shown in Figure 2(b).

2.5 | In vivo live mouse measurements

2.5.1 | Subjects

Adult male Balb/c mice were supplied from the Animal Resources Centre (Murdoch, WA, Australia) housed in 18°C ± 6 with 12:12 h light: dark and with food and water provided ad libitum. All procedures were conducted under the University of Adelaide Animal Ethics approval number M-2018-024 in compliance with the Australian code for the care and use of animals for scientific purposes.

2.5.2 | Surgical procedure

Under deep anaesthesia with 2% inhalation isoflurane and animal in ventral recumbency, the lower thigh area was prepared aseptically for an unrelated surgical procedure. A subcutaneous pocket was created via an 18-gauge needle insertion in the semitendinosus muscle. Next, the tip sensor was implanted in the muscle mass via the inserted needle for the duration of the experiment. To induce hypoxia, the inhalation isoflurane was increased to 5% and the oxygen level was reduced to a minimal level; protein carbonyl sensing continued for the remainder of the experiment until the time of death. The same procedure was done in subcutaneous lumbar area.

2.6 | Data analysis

These dynamic studies are based on repeated observations within an individual sample over time. Linear regression modelling was used to find the sensor signal vs concentration dependence. Fickian diffusion mathematical model was used for dynamic diffusion results. Other dynamic results shown within skin and small animal models are limited to demonstrate the ability of the tip sensor to perform dynamic in vivo measurements. Statistical analysis of small animal models would require a greater number of animals and so is beyond the scope of this study.

3 | RESULTS AND DISCUSSION

3.1 | Sensor characterization

No fluorescence was observed when the tip sensor was placed in water only. However, intense fluorescence with a peak at 517 nm formed within 2 min of placing the tip sensor in a 0.5 mM concentration of BHB, as shown in Figure 3.

To test the stability and reversibility of the tip sensor while obtaining a standard curve, measurements were performed every minute over a 1-h period using decreasing concentrations of the

| TABLE 1 BHB solution preparation |
|----------------------------------|
| Concentration | Buffer added | Total volume |
|----------------|---------------|--------------|
| 0.5 mM         | –             | 200 μl       |
| 0.4 mM         | 50 μl         | 250 μl       |
| 0.3 mM         | 83 μl         | 333 μl       |
| 0.2 mM         | 167 μl        | 500 μl       |
| 0.1 mM         | 500 μl        | 1000 μl      |
BHB stock solutions described in Section 2.3. The intensity of the fluorescence peak at 517 nm was found to be proportional to the concentration of BHB following the equation of $S = 27174223 \times C$, where $S$ is the intensity in counts and $C$ is the Molar concentration. This linear dependence is shown by the red dotted line in Figure 4, along with the unadjusted peak counts measured from one pulse every minute with the sensor in the BHB stock solutions with decreasing concentrations every 10 min. The last measurement was performed in buffer only. The tip sensor was further characterized in a mouse embryo cell growth assay to ensure its biological compatibility, which showed no affects to the viability and growth of the cell culture (mouse embryo assay).

### 3.2 | In vivo porcine skin measurements

Fresh porcine skin was used to further characterize the tip sensor. To achieve this, the tip sensor with microneedles shown in Section 2.4 was used for subcutaneous measurement. A microneedle length of 5 mm was chosen so that the tip of the needles would penetrate the epidermis and dermis layers of the porcine skin to locate within the subcutaneous tissues (Lonergan et al., 2019; Turner et al., 2015). The microneedle array consisted of two needles, one of which was used for the fibretip sensor and the other needle contained a capillary so that sample analyte or medications could be injected alongside the sensor. The microneedle was inserted into three different locations of fresh (3 h old) porcine skin obtained from an abattoir on the morning before the experiment; measurements were performed every minute. Results from the tip sensor, shown in Figure 5, indicated that the protein carbonyl concentrations were significantly different at these three locations, with an increased concentration detected where there was no blood in the sample.

The signal was an order of magnitude higher in regions of the skin where blood was not noticeably present. One possible explanation for this is that protein carbonyl levels formed earlier in response to oxidative stress could be more stable in subcutaneous tissue compared to blood. The same experiment was repeated on 9-day-old...
porcine skin, where the biosensor showed very low intensity; orders of magnitude less signal compared to the 3 h old sample. The results also show that it took around 2 min for the tip sensor to respond to changes in concentration of protein carbonyls, each time the tip sensor was inserted into the porcine skin.

3.3 Subcutaneous tissue diffusion measurements

The efficient application of subcutaneous injection of drug solution relies on a detailed understanding of the mass transport of substances in surrounding biological tissues upon release. Experimental techniques capable of characterizing these transport processes in vivo are highly needed in the design and development of controlled release delivery systems. The microneedle array was designed with a 3 mm gap between the capillary injection needle and the tip sensor needle to measure the dynamic concentration flux dynamics within the subcutaneous tissue. These types of measurements can help with understanding the mechanical and diffusion properties of subcutaneous tissue with the potential to avoid adverse effects of subcutaneous delivery treatments. The measurements were achieved by inserting the microneedle array into 10-day-old porcine skin and taking measurements every minute from the tip sensor within the microneedle array while pumping (LongerPump BT100-1F) BHB at a controlled flow rate through the capillary side of the microneedle array. For the first forty minutes, a 5 μL/min flow rate of 1 mM BHB solution was applied to the capillary side of the microneedle array. The intensity of the fluorescence peak at 517 nm from the tip sensor steadily increased with a slight bell curve to a maximum 16 thousand counts, as shown in Figure 6. Next, the tip sensor was removed and reinserted into a fresh part of the porcine skin sample and a 20 μL/min flow rate of 0.5 mM BHB solution was applied for the next 35 min to the capillary side of the microneedle array. The intensity of the tip sensor initially reduced to around three thousand counts and then steadily increased to around eight thousand counts with a well-defined bell curve shape. Finally, the tip sensor was again removed and reinserted into a fresh part of the porcine skin sample and a 5 μL/min flow rate of 0.5 mM BHB solution was applied for the next 50 min to the capillary side of the microneedle array. The intensity of the tip sensor again initially reduced to around three thousand counts and then steadily increased to around 7 thousand counts with a well-defined bell curve. The rise time of the tip sensor response to the 5 μL/min flow rate was slower compared with the rise time of the 20 μL/min flow rate, at the same concentration of 0.5 mM BHB. The starting point for all these measurements was approximately one thousand counts, which is expected to be because of the residual concentration of protein carbonyls in the subcutaneous tissue.

Diffusion in the subcutaneous tissue is modelled by Fick’s law (Crank, 1979), which in one dimension can be described by

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \tag{1}$$

where x is the distance, t is the time, C refers to the concentration of the injected analyte, and D is the analyte diffusivity through the subcutaneous tissue (diffusion coefficient). D is assumed to be constant in the concentration range investigated. We have the boundary conditions that at $t = 0$, $x = 0$ and $C = C_0$. Using the infinite approximation, a solution of Equation (1) is as follows (Crank, 1979):

$$C(x, t) = \frac{C_0}{2} - \frac{C_0}{\sqrt{\pi}} \int_0^t \frac{e^{-\frac{x^2}{4Dt}}}{{\sqrt{t}}} dt \tag{2}$$

The right side of Equation (2) is the familiar error function which can be integrated using a power series expansion. Using the first two terms of the power series expansion as a quick approximation with the assumptions that $x > 0$ and relatively small (0.003 m), $t > 0$ and relatively large (more than 180 s), Equation (2) becomes:

$$C(x, t) = C_0 \left( \frac{1}{2} - \frac{12Dt - x^3}{24Dt^\frac{3}{2}} \right) \tag{3}$$

Equation (3) was fitted to the data shown in Figure 6 to determine the diffusion coefficient D, as shown by the narrow dark results for each experiment. The points for $t < 180$ s are not shown as it was found that the result from Equation (3) was significantly inaccurate. More terms of the power series expansion would need to be included if the model needed to be used where $x > 0.003$ m or $t < 180$ s. While Equation (3) fitted well to both 0.5 mM results, the 1 mM results deviated from the expected error function curve which might be due to leakage or backflow during the experiment.
However, the model did fit very well ($R^2 = 0.952$) to the first 22 min of the 1 mM experiment. The results of the fitting (Table 2) indicate that $D = 1.5 \times 10^{-8}$ at a flow rate of 5 $\mu$l/min, as shown by 1 mM and 0.5 mM results. At a flow rate of 20 $\mu$l/min, $D$ increased by more than twice to $3.7 \times 10^{-8}$. In the future, it might be useful to perform a more extensive set of experiments that include a larger set of flow rates and concentrations to better understand how the diffusion coefficient of subcutaneous tissue varies with flow.

### 3.4 | In vivo mouse measurements

Next, the biosensor was used to measure protein carbonylation levels in an anesthetized live mouse (as described in Section 2.6). In this case, the polymer microneedle was replaced by a stainless-steel hypodermic needle. The tip sensor was placed in the subcutaneous space under the skin via the hypodermic needle for the first 12 min and then placed in the hind leg muscle for the remaining 10 min. The results revealed a detectable and relatively steady concentration of the analyte in the subcutaneous space under the skin, as shown in Figure 7. In the hind limb muscle, where there was blood, the levels were much higher and seemed to increase over the 10 min of measurement. While an increased level was expected in the hind leg muscle (Weber et al., 2015) and considering this is the first time such a measurement was made on a living model; more work is needed to understand why the biosensor in the hind leg muscle measured an order of magnitude higher signal compared with subcutaneous measurements from the mouse or pig skin.

The protein carbonyl levels were measured in the subcutaneous space under the skin of another two mice. This time the experiment was extended to 1 h. As was the case for the first mouse, the result from the sensor in the second mouse also increased for the first few minutes after insertion. However, the measurement then decreased back to a relatively steady state after 20 min, as shown in Figure 8. Using the concentration equation discussed in Section 3.1, we find that the concentration of protein carbonyls becomes steady at around 36.8 $\mu$M. It is expected that this initial increase is due to immune response from inserting the needle and sensor, and that response quickly subsides as the sensor is left in place. This was also the steady-state protein carbonyl concentration of the third mouse after 20 min. Unexpectedly, the third mouse did not have the same initial increase as measured from the first two mice, as shown in Figure 8. Further experiments with increased sample size and for longer periods of time are needed to understand how dynamic concentration changes are related to specific stresses and to test the reasons for the unexpected result from the third mouse.

### Table 2 Results of Equation (3) fitted to data in Figure 6

| Concentration BHB | Flow rate ($\mu$l/min) | $D$ ($m^2/s$) | $R^2$-square |
|-------------------|------------------------|--------------|-------------|
| 1 mM              | 5                      | $1.5 \times 10^{-8}$ | 0.952       |
| 0.5 mM            | 20                     | $3.7 \times 10^{-8}$ | 0.9151      |
| 0.5 mM            | 5                      | $1.5 \times 10^{-8}$ | 0.9153      |

### 4 | CONCLUSIONS

This paper describes a new method for measuring in vivo dynamic protein carbonylation and initial results of those measurements.
Using the small calibrated optical sensor has made dynamic measurements possible for the first time, which is far more advantageous than in vitro measurement requiring sample preparation. While there is considerable evidence on the involvement of oxidative stress leading to protein carbonylation in a number of diseases and conditions, there are still many questions that need to be explored about how oxidative stress is involved in health. The study of oxidant/antioxidant dynamics has the potential to contribute to the understanding of important pathways involved in regulation of cellular functions and metabolism. With this new measurement tool, future research now has the ability to investigate the dynamics involved in the maintenance of redox homeostasis especially during particular physiological conditions when metabolism is significantly increased, and homeostatic mechanisms are challenged. This new sensing technology also has the potential to provide critical decision support information about oxidative stress levels, which could improve outcomes for people living with chronic disease and age-related disorders, as well as provide crucial redox balance information for athletes and livestock, and ultimately improve our understanding about the mechanisms affecting overall health.

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