

Microfluidic perfusion system for maintaining viable heart tissue with real-time electrochemical monitoring of reactive oxygen species†

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A microfluidic device has been developed to maintain viable heart tissue samples in a biomimetic microenvironment. This device allows rat or human heart tissue to be studied under pseudo in vivo conditions. Effluent levels of lactate dehydrogenase and hydrogen peroxide were used as markers of damaged tissue in combination with in situ electrochemical measurement of the release of reactive oxygen species (ROS). The parameters for perfusion were optimized to maintain biopsies of rat right ventricular or human right atrial tissue viable for up to 5 and 3.5 hours, respectively. Electrochemical assessment of the oxidation current of total ROS, employing cyclic voltammetry, gave results in real-time that were in good agreement to biochemical assessment using conventional, off-chip, commercial assays. This proof-of-principle, integrated microfluidic device, may be exploited in providing a platform technology for future cardiac research, offering an alternative approach for investigating heart pathophysiology and facilitating the development of new therapeutic strategies.

Introduction

Studies of cardiac physiology and biochemistry have traditionally used in vitro perfusion systems such as the Langendorff retrograde perfusion.^{1,2} This model has been widely used for a diverse range of studies of the heart, including molecular and cellular alterations, as well as evaluating therapeutic interventions.

The main alternative approaches for studying the pathophysiology of heart diseases are based on cell or tissue culture. However, the concern for all in vitro cell manipulation studies is that the cells behave differently in culture flasks,³ as the microenvironment experienced in vitro is clearly different from those in vivo. In addition, there is a further complication with adult cardiomyocytes as these can only be maintained as primary cell cultures, not immortal cell lines. Tissues and organs have complex three-dimensional systems in situ, including a complex network of extra-cellular matrix, e.g. elastin, laminin, collagen and fibronectin. In addition, cells inhabit a closely packed spatio-temporal environment where any cell is always communicating and interacting with multiple other cell types, responding to local concentrations of a plethora of molecules, such as cytokines, enzymes and nutrients. Accordingly in an in vitro cell culture environment, many, if not all of these signals are missing.

Microfluidics offers an approach that can circumvent many of the limitations of in vitro cell culture methodology. For example, by continuously supplying enriched media to heart tissue biopsies in vitro, whilst removing waste products and gases produced by cellular processes.^{4,5} Several groups have developed microfluidic devices for studying cardiomyocyte functions, with Li and Li being the first, investigating cell contraction.⁶ A single cardiomyocyte was retained within a V-shaped structure of the chip. Analysis of intracellular $[Ca^{2+}]$ following ionomycin stimulation was measured using the calcium-sensitive dye, Fluo-4 AM ester. Subsequently, Li et al. developed a microfluidic chip with improved cell retention properties and used this to monitor calcium mobilization in single cardiomyocytes, in real-time, as a part of a drug screening application.⁷ Cheng et al. developed a multi-functional microfluidic platform with a microelectrode array to stimulate the cell and monitor lactate release by electrochemical (EC) probes.⁸ This analysis was used in conjunction with in situ microscopy and fluorescence detection of extracellular pH and cellular Ca^{2+} concentrations during cell contraction. However tissue, with its highly complex structures, presents a greater technological challenge in terms of maintaining essential supplies of

oxygen and nutrients, whilst removing waste products. The benefits of studying a more holistic representation of the complex cell–cell and cell–stroma interactions are a greater relevance for physiological and clinical studies.

Reactive oxygen species (ROS) play a major role in ischemia–reperfusion injury, ageing, neurodegenerative disorders and many other diseases.^{9,10} ROS can cause the oxidation of membrane phospholipids, DNA and proteins, subsequently impairing mitochondrial function. ROS can be measured by various means, such as chemiluminescence,^{11,12} fluorescence,^{13,14} infrared¹⁵ and electron spin resonance spectroscopy.¹⁶ Electrochemical methods, however, provide a direct means of analysing the concentration of free radicals in solution at the specific site where the sensing electrode is positioned, with minimal disturbance to the sample under investigation.¹⁷ Fast cyclic voltammetry has been carried out previously to capture the transient signals of extracellular nitric oxide or nitric oxide synthase (NOS) in brain slices.^{18,19} Recently, triple potential-step chronoamperometry has been developed to measure simultaneously ROS and reactive nitrogen species (RNS).²⁰

Several groups have reported electrochemical sensors integrated with microfluidic devices to determine changes in ROS. A microfabricated Pt electrode was platinised to increase electrochemical sensitivity and used to detect ROS and RNS released from macrophage cells following continuous microinjection of a calcium ionophore.²¹ Electrochemical measurement has also been used to detect lactate released from heart cells⁷ and in saliva.²² Hitherto, electrochemical monitoring of ROS during perfusion of viable heart tissue biopsies has not been reported.

Here, a microfluidic chip based perfusion system has been designed and optimised to maintain viable and functioning heart tissue samples. Analysis of tissue was performed on-chip by electrochemical measurements and verified off-chip by biochemical methods, as a route towards developing the next generation of user-friendly devices to maintain and analyse tissue to address clinically relevant issues.

Experimental

Experimental models

All procedures conform to the UK Animals (Scientific Procedures) Act 1986. Wistar rats (B&K Universal Ltd., Grimston, UK) were housed under a 12:12 hour light–dark cycle and provided with food and water ad libitum. Animals were anaesthetized via intraperitoneal injection of 0.5 mL 100 g⁻¹ sodium thiopentone (25 mg mL⁻¹, Link Pharmaceuticals Ltd., West Sussex, UK). In brief, hearts were rapidly excised and the aorta was cannulated. A piece of right ventricular tissue (approximately 2 × 4 × 4 mm³) was taken and placed directly into the chamber of the perfusion device filled with buffer. At the end of the experiment, the wet weight of the heart tissue sample was recorded.

Human heart tissue biopsies were supplied by Mr S. Griffin (Consultant Cardiothoracic Surgeon). Ethics and Hospital Trust approvals were obtained from Hull and East Yorkshire Local Research Ethics Committee (07/H1304/105) and Hull and East Yorkshire NHS Trust (R0568), respectively. Samples were taken from patients undergoing coronary artery bypass surgery (CABG). Heart biopsies were immersed in freshly prepared cardioplegic solution at 4 °C (Martindale Pharmaceuticals, UK), transported to the laboratory and mounted in the perfusion chamber within 60 minutes.

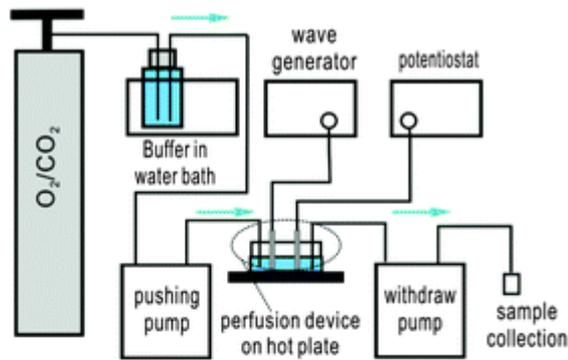
Fabrication of microfluidic perfusion device

A microfluidic perfusion chamber with a capacity of 400 μL was constructed from polydimethylsiloxane (PDMS) (Dow Corning, USA) and a polystyrene Petri dish (35 mm in diameter) (Fig. 1). Briefly, the chamber was moulded around the diamond-shaped end of a 1 mL syringe barrel that was attached to a silicon wafer using an instant adhesive (Henkel Loctite Adhesives Ltd., UK). Mixed and degassed PDMS monomer and initiator (w/w, 10 : 1) were poured over the syringe end and cured at 75 °C for two hours to form a mould. The PDMS chamber was stripped off the template and five holes (1–3 mm in diameter) were punched to allow for the inlet, and outlet Teflon™ tubings (1.0 mm ID × 1.6 mm OD, VWR Int. Ltd.), and electrodes. The PDMS was bonded with the Petri dish after oxygen plasma treatment at 350 V,

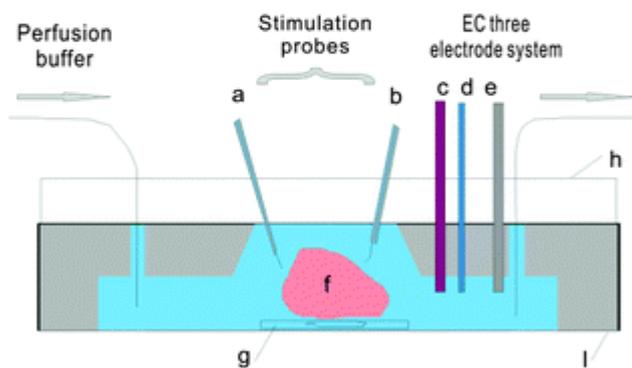
20 mA, P_{O_2} 8 mbar for 50 s (SPEEDIVAC, Model 12E6/1405 Edwards High Vacuum Ltd., UK). This device provided a 7 mm chamber at its centre into which a heart biopsy was placed and positioned on a 37 °C hotplate (WIS1, World Precision Instruments, UK). Both the inlet and outlet were connected to peristaltic pumps at a flow rate of 120 $\mu\text{L min}^{-1}$, and the heart tissue was electrically stimulated via two platinum wire electrodes anchored in the chamber lid using a programmable, square wave, function generator (TG1010A, Thurlby Thandar Instruments Ltd., UK).

Fabrication of microfluidic perfusion device

(A)



(B)



(C)

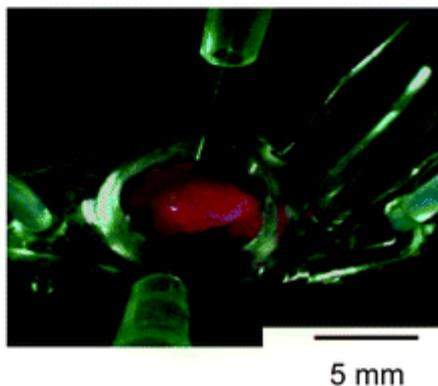


Fig. 1 Schematics of the experimental setup (A and B): stimulation electrodes (a and b); working, reference and counter electrodes (c–e); tissue in chamber with Krebs–Henseleit buffer (supplemented with 5 mM glucose, 100 Units mL^{-1} penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin, f); tissue holder (PDMS, g). Petri dish lid as the chamber cover and holder for electrodes and tubes (h). Polystyrene Petri dish (i). Photograph of the device with tissue (without the chamber lid) (C).

Experimental protocols

Prior to each experiment, the microfluidic system was sterilised with 70% (v/v) ethanol/water by perfusion at $120 \mu\text{L min}^{-1}$ and rinsed with sterilised double distilled water for 10 minutes, respectively. The microfluidic device was then primed with Krebs–Henseleit buffer (KH, 118 mM NaCl, 25 mM NaHCO_3 , 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgCl_2 and 2.5 mM CaCl_2) for 15 minutes. In this paper KHG represents KH buffer plus 5 mM glucose. The addition of 100 Unit mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin (final concentration) to KHG buffer formed KHGB. All constituents were purchased from Sigma-Aldrich (UK) in the highest purity grade commercially available and used without further purification. Buffers were filtered using a $0.22 \mu\text{m}$ syringe filter (Millipore Corporation, USA) and oxygenated with 95% O_2 -5% CO_2 continuously at 37°C .

Right ventricular tissue from rat or right atrial tissue biopsies from patients were perfused with KHGB for up to 5 hours followed by 30 minutes to one hour with 2% (v/v) Triton X100 (BDH, England) in KHGB. Triton X100 is a non-ionic surfactant able to solubilise phospholipid membranes,²³ releasing the intracellular enzymes. In some experiments, 95% N_2 -5% CO_2 saturated KHGB was used as an alternative method of inducing damage.

Assessment of tissue functions

1 Tissue viability. Viability was evaluated using calcein–acetoxymethyl ester (Calcein AM; Invitrogen, UK) and propidium iodide (PI; Sigma-Aldrich, UK). Calcein AM is a modified carboxylic acid containing an additional ester group that permits entry into cells. The ester is hydrolysed within viable cells altering hydrophobicity; the resulting carboxylate is both fluorescent and membrane impermeable. Conversely, PI only enters cells when the plasma membrane is disrupted where it intercalates with the double-stranded DNA and fluoresces, thus providing a measure of dead or dying cells. Following perfusion for various time periods, cardiac tissue sections were incubated with $50 \mu\text{L}$ of $10 \mu\text{M}$ calcein AM ($\lambda_{\text{ex}} = 494 \text{ nm}$ and $\lambda_{\text{em}} = 517 \text{ nm}$) in phosphate buffered saline (PBS, pH 7.4) for 1 hour. The tissue was then washed with PBS and incubated with $50 \mu\text{L}$ of 3.75 mM PI ($\lambda_{\text{ex}} = 536 \text{ nm}$ and $\lambda_{\text{em}} = 617 \text{ nm}$) in PBS for 10 minutes. After a final wash with PBS, the cardiac tissue section was examined under a laser-scanning inverted confocal microscope (Nikon Eclipse TE2000-E).

2 Lactate dehydrogenase measurement. Cell damage was determined via release of lactate dehydrogenase (LDH),²⁴ using a commercial colorimetric assay (Cytotoxicity Detection Kit^{Plus}, Roche, UK). Effluent samples were collected for 2 min (approximately $240 \mu\text{L}$ in total) at 5 min intervals during perfusion from the outlet tube (see Fig. 1(A)). To measure LDH, samples ($50 \mu\text{L}$) were incubated with $50 \mu\text{L}$ of reaction mixture containing diaphorase/ NAD^+ , iodotetrazolium chloride and sodium lactate for 30 min at room temperature in 96-well plates. The reaction was terminated by adding $50 \mu\text{L}$ of stop solution (1 M HCl) per well, and absorbance measured at 492 nm using a microplate reader (BioTek Instruments, Inc., USA). All samples were assayed in triplicate.

3 Detection of H_2O_2 . The concentration of H_2O_2 in the effluent was determined using a commercial Amplex Red Hydrogen Peroxide assay kit (Invitrogen Molecular Probes, UK). A standard curve ranging from 0 to $5 \mu\text{M}$ H_2O_2 was generated for each experiment. Samples ($50 \mu\text{L}$) were incubated with $50 \mu\text{L}$ of reaction mixture containing $100 \mu\text{M}$ Amplex Red reagent and 0.2 U mL^{-1} horseradish peroxidase (EC. 1.11.1.7, one unit of enzyme will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20°C) for 30 minutes at room temperature. Absorbance at 562 nm was measured using a microplate reader (BioTek Instruments Inc.). All results were corrected for background absorbance and expressed as the average of duplicate samples.

4 Electrochemical monitoring of the total ROS. Electrochemical measurements were performed with the three-electrode system integrated onto the perfusion chip by a PalmSens potentiostat (PalmSens Instruments, Netherlands). This in-house produced system comprised one Pt disk electrode ($250 \mu\text{m}$ in diameter) sealed in a glass tube (3 mm OD) which served as a working electrode (WE), a Pt wire as a counter electrode and Ag wire with a layer of AgCl operating as a reference electrode. Note that the saline buffer is sufficient to ensure that this reference maintains a constant potential. The AgCl layer was electrochemically coated by placing Ag wire in 100 mM NaCl solution and holding the potential at 0.45 V vs. Ag/AgCl (3 M NaCl) for 10 min until the oxidation current tailed to a residual. Potentials are reported versus this reference electrode unless otherwise stated. Prior to use, the working electrode was polished with $0.3 \mu\text{m}$ and $0.1 \mu\text{m}$ alumina slurry on polishing pads (Kemet International Ltd., Kent, UK),

respectively and then successively cleaned in 10% (v/v) HNO₃ and water in an ultrasonic bath. During tests, refreshing of the electrode surface was carried out by wiping the WE on a polishing cloth and rinsing with water. During in situ measurements, cleaning of the WE was carried out by electrochemical methods, such as differential pulse voltammetry (DPV) or cyclic voltammetry. During perfusion, cyclic voltammetry was conducted between -0.8 V and 1.0 V at a scan rate of 0.1 V s⁻¹ and followed by a square wave voltammetry (SWV) scan from -0.55 V to 1.0 V with a frequency of 25 Hz, amplitude of 25 mV and a step of 5 mV. The first scan of cyclic voltammetry data was used for “non-polishing-mode” analysis while the second scan was used when polishing was undertaken, due to the enhanced sharpness of the feature in the second cycle. The electrochemical measurement was carried out when the stimulation pulse (E_s) was switched off to obviate the deterioration in voltammograms.

Results and discussion

Perfusion system and viability assessment of tissue

1 Establishment of an optimal perfusion system. The dimensions of the tissue samples were optimised so that sufficient oxygen was supplied to the heart tissue sample. Samples with dimensions of approximately 4 × 2.5 × 2 mm³ were evenly labelled with calcein AM with little PI staining after perfusion for 5 hours (Fig. 2(A)). Larger heart tissue biopsies had a greater distance for oxygen and nutrient permeation from the edge to the core of the tissue sample; and thus the central region was strongly stained with PI but with a reduced calcein signal (Fig. 2(B)). Flow rates <100 μL min⁻¹ meant that the collection of sufficiently sized aliquots took too long for repeated analysis, whereas flow rates ≥200 μL min⁻¹ disturb the tissue and stimulation probes preventing reproducible assessment; see later and ESI† for EC optimisation. A flow rate of 120 μL min⁻¹ was established as the standard flow rate for all subsequent experiments.

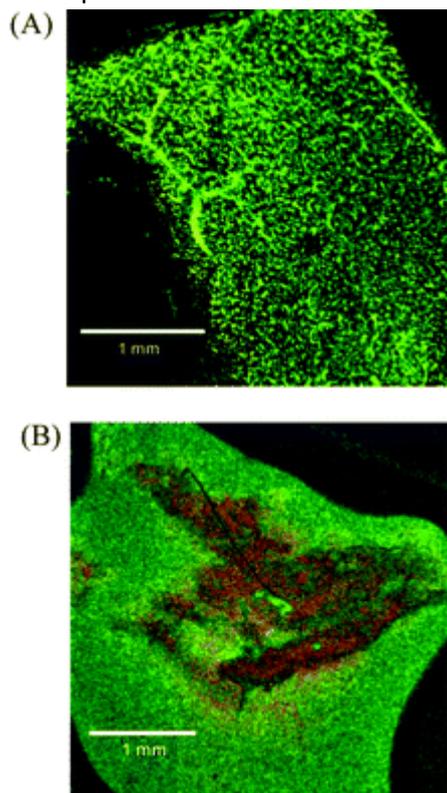


Fig. 2 Representative confocal microscopy images of cardiac tissue sections treated with calcein AM (green fluorescence) and PI (red fluorescence) following 5 h incubation and an hour treatment with 2% (v/v) Triton X100. Tissue size in (A) ~4 × 2.5 × 2 mm³; (B) ~5 × 3.5 × 2 mm³. A 10× objective lens was used. The images were 400–500 μm from the bottom surface of the tissues.

2 Establishment of on-chip electrical stimulation parameters. The heart tissue in the current study was stimulated electrically to mimic the in vivo situation. It was found that the excitation threshold for rat tissue to start beating and be maintained for at least 1 min was 1.0 V cm⁻¹ at 2 Hz. Normally, the stimulation regime using square monophasic pulses, for a whole rat heart, occurs for a duration of 5 ms at a

frequency of 5 Hz and amplitude of 1.5–2 V;²⁵ or for approximately 3 ms at a frequency between 0.5 and 1 Hz with a field strength of 5–6 V cm⁻¹ for single rat ventricular cardiomyocytes.^{26,27} For rat tissue samples of the sizes described in Fig. 2 stimulation started at 0.8 V cm⁻¹ and 2 Hz and the electric field was incrementally increased until all or part of the tissue commenced beating. At 1 V cm⁻¹ the beating was observed to last more than 1 min. Bubbles due to solvent breakdown formed when the electrical field was ≥ 12 V cm⁻¹. A standardized frequency of 1.5 Hz with an electric field of 3–4 V cm⁻¹ was chosen in order to obtain prolonged heart beating.

The contractile function of tissue in the perfusion device became more pronounced, and generally lasted for longer periods of time when the calcium concentration in the KH buffer was increased from 1.25 to 2.5 mM. The latter concentration was used as standard for all subsequent experiments.

The optimized conditions (3–4 V cm⁻¹, 1.5 Hz, 2.5 mM calcium chloride) maintained the heart tissue with regular contractions for up to 5 hours. Contraction of the electrically excited heart tissue sample was observed via a microscope camera (Veho, UK) during the perfusion of the majority of rat tissue biopsies, but only about 40% of human tissue samples showed this trend. The key determinant of damage was LDH release, measured in effluent samples and following incubation of the tissue with Triton X100 at the end of the perfusion period in the device. An alternative method of inducing injury was through perfusion with media containing 95% N₂-5% CO₂. It was observed that rat tissue ceased functioning within 10 min after incubation in KHG buffer saturated with this gas, associated with a marked increase in LDH release, similar to that observed after incubation with Triton X100 (Fig. 3).

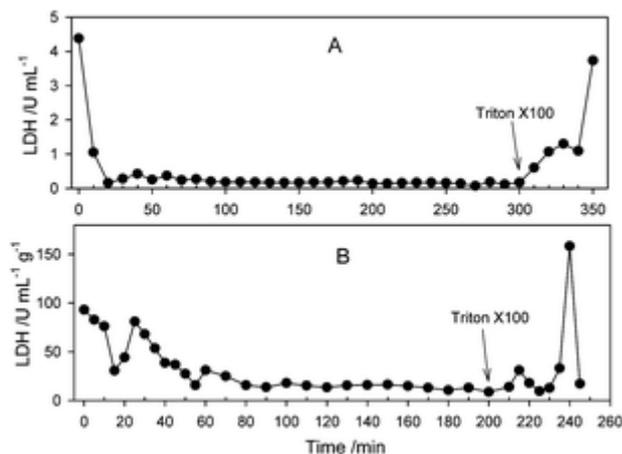


Fig. 3 LDH level in rat (A) or human (B) tissue. Tissues were perfused with oxygenated KHGB until 300 min in (A), and 200 min in (B), followed by 2% (v/v) Triton X100 in KHGB. Stimulation pulse, 4 V cm⁻¹; 1.5 Hz. The LDH concentration was calculated in U mL⁻¹ in (A) and subsequently standardised against the wet weight of human tissue (B). (Representation of three and eleven experiments in (A) and (B), respectively.)

3 Viability in perfusion device as assessed by LDH release. Biopsies from right ventricular rat tissue were maintained in a viable state for at least 5 hours (Fig. 3A), whereas for human right atrial tissues, a period of 3.5 hours was the maximum tested (Fig. 3B). LDH activities were high initially in every experiment, most likely due to damage caused when samples were taken and transported to the microdevice. Following this initial peak, the LDH levels remained at low levels, suggesting a recovery from the acute injury due to sufficient supplies of nutrients, oxygen and appropriate electrical stimulation in the flow perfusion chamber. When oxygen was removed from the buffer, electrical stimulation was stopped, or the biopsy was treated with Triton X100, the LDH levels showed a sharp increase within 10–15 minutes, verifying the viability of tissue prior to induction of damage.

The current work here is distinct from tissue engineering constructs in microfluidic systems, which generate new tissues to restore, maintain, or enhance tissue function. The methodology described here allows direct experimentation on primary clinical samples under in vivo conditions. Similar work has been demonstrated by our group using liver tissue²⁸ and colorectal tumour biopsies,²⁹ both of which were kept

viable for over 70 hours in the microfluidic environment. The liver tissue produced albumin and urea during perfusion, whereas the colorectal tissue produced vascular endothelial growth factor in response to hypoxia, demonstrating that both cell types were functional and responded in a similar manner to tissue *in vivo*.

ROS monitoring by electrochemistry

ROS or NOS can be measured by various kinds of chemical means.^{11–16} Electrochemical measurement has distinct advantages in terms of sensitivity and speed for real-time assessment, *in situ*. Previously H_2O_2 has been reported to be released at a rate of $50 \text{ nmol g}^{-1} \text{ min}^{-1}$ two minutes after an ischaemia/reperfusion episode.³⁰ Following previous work in our laboratory regarding the development of a hydrogen peroxide biosensor, preliminary studies indicated that this enzyme-modified electrode could be employed to measure H_2O_2 using the current methodology.³¹ Given that the half-life of H_2O_2 is longer than that of the other ROS and NOS, we chose to employ direct H_2O_2 oxidation at Pt electrodes as the validation of part of ROS.

1 Species confirmation and identification of the monitoring window. Details regarding the amperometric monitoring of ROS are given in ESI 1†; in summary it was observed that potentials of +0.72 V vs. Ag/AgCl allowed for the oxidative detection of ROS.^{21,32}

2 Control tests. Monitoring the current change continuously in the perfusion system is a challenge because many parameters can cause interference with the electrochemical measurements. The cleanliness of the WE, the presence or absence of oxygen and nitrogen in different stages of the perfusion, Triton X100 and the stimulation for maintenance of the heart “beat” were the main interfering parameters.

Two modes of control test for EC measurement were studied: non-polishing and polishing of the electrode in the relevant buffers. In the first mode, the WE was scanned initially in KH solution from -0.8 V to 1.0 V until a stable response was observed; the electrode remained in place within the chamber during perfusion without cleaning prior to voltammetric measurement. In the second control the WE was polished and rinsed with water before each measurement; Fig. 4 illustrates these results. It was found that the “on/off” stimulation pulse caused fluctuations in the background current measured using cyclic voltammetry or SWV. When no stimulation pulse was applied, the baseline was relatively noise-free and stable with a low standard deviation (Fig. 4a, and ESI 2, Table S1†).

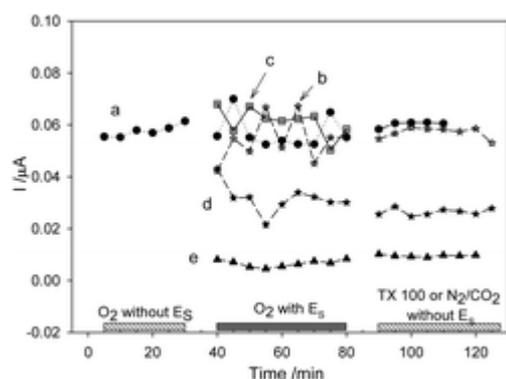


Fig. 4 Control tests in different buffer solutions using polished electrodes. Between 0–30 min and 90–125 min, no stimulation pulse; 40–80 min, stimulation pulse on while not conducting EC measurement. I_0 at 0.72 V in cyclic voltammetry in KHGB buffer with Triton X100 from the 90th min (a, solid circle), in KHG with 95% N_2 -5% CO_2 from the 90th min (b, empty star) and in KH (c, square-cross). I_0 at 0.3 V in SWV with 95% N_2 -5% CO_2 from 90 min, in KHG (d, solid star), and KHGB (e, solid triangle). Flow rate: $120 \mu\text{L min}^{-1}$ for all perfusions. (Representative of three independent experiments.)

Higher variances of oxidation current (I_0) were observed when electrical stimulation was applied, as expected (region 40–80 min in Fig 4). Glucose and antibiotics were not responsible for this because further tests in KH and KHG buffers suggested a similar fluctuation within this period (Fig. 4b and c). When electrical stimulation is applied ionic species will redistribute around the pulse probes to form electrical

double layers, which may alter the background current of a voltammetric measurement each time this stimulation is turned on. In order to mitigate this effect EC measurement was delayed for 5 seconds after the pulse ceased to ensure sufficient time for the double layer around the electrode to reset in the presence of freshly flowing buffer.

3 Study of induction of injury and in situ EC measurement. In Triton X100–KHGB with no electrical stimulation (90 to 125 min), the background current I_0 at 0.72 V in cyclic voltammetry fluctuated by up to $\pm 10\%$ compared with those between 40 and 80 min without Triton X100; whilst in 95% N_2 -5% CO_2 –KHGB, I_0 increased less than 6% on average (Fig. 4). The surfactant was reported to enhance efficiently the electron transfer between solution and electrode surface due to its strong adsorption on the hydrophobic electrode surface.³³ Since the use of Triton X100 in tissue-free buffer caused an increased signal of up to 10%, the tissue would be considered alive before the assault only when the current measured in EC increased by more than 15%.

In order to achieve in situ EC measurement, a non-polished electrode was first used. The baseline was found to be smooth and stable when there were no electrical stimulation periods but suffered poor reproducibility and a decreasing baseline when stimulation pulses were switched on. The average I_0 decreased more than 20% with addition of Triton X100, and the relative standard deviation was 12% on average. The WE also became easily contaminated during voltammetry scans. This was confirmed by using a Pt disk electrode in N,N,N',N'-tetramethyl-p-phenylenediamine solutions (TMPD, Sigma) before and after cyclic voltammetry scans in KHGB and KH (ESI 3, Fig. S2†). Distorted voltammograms were observed in the range of 0.05 V to -0.02 V in the TMPD solution after scans in KH buffers. However, when operating in the non-polishing mode, it was necessary to minimise disturbances of the device once established, hence a method for cleaning the electrode in situ by electrochemistry was designed. DPV and cyclic voltammetry, which scan to high potentials (from -0.5 V to 1.5 V with the E_s -on), were demonstrated to clean the WE effectively in situ, when fouling was observed by increased reduction currents around 0.8 V in cyclic voltammograms.

In order to increase the sensitivity of the EC measurement and shorten the record time, thus limiting any potential disruption of the perfusion, measurement of ROS by SWV was also studied. Generally, the average I_0 at 0.25 V in SWV was smaller than that in cyclic voltammetry (Fig. 4 (d, KHG) and (e, KHGB)). This lower current at 0.25 V in KHGB is due to the presence of antibiotics ((d) in Fig. S1A, inset†), which may induce electrode surface blocking after the SWV scan. This can be found in 90–125 min area when 95% N_2 -5% CO_2 took the place of O_2 and electrical stimulation was off; the standard deviation was better than in cyclic voltammetry, whilst the variance of I_0 after N_2 treatment was $\pm 20\%$, higher than in cyclic voltammetry.

4 ROS results compared with LDH and H_2O_2 assays. Fig. 5A shows the results of LDH and in situ EC measurement of ROS in real time. The electrode was cleaned by the EC method described above. The profile of LDH release (measured off-chip) and EC agreed well, with relatively high levels in the early stage of the perfusion, this then decreased and remained low until a sharp increase was induced with the addition of Triton X100. The electrode was cleaned by DPV with scans from -0.8 V to 1.5 V before the 25th, 55th, 140th and 190th min, when the WE was contaminated (an early sign was increased cathodic current around 0.8 V in cyclic voltammetry). The decrease of signal at the 55th and 190th min accordingly is likely to be due to this action. It was noted that the length of time between obtaining the biopsy and placing this in the chamber was correlated with electrode contamination, i.e. when placed in the device within 5 minutes, little electrode contamination was observed; unfortunately the human tissue had to be transported from the hospital site and took up to 60 min from biopsy to device.

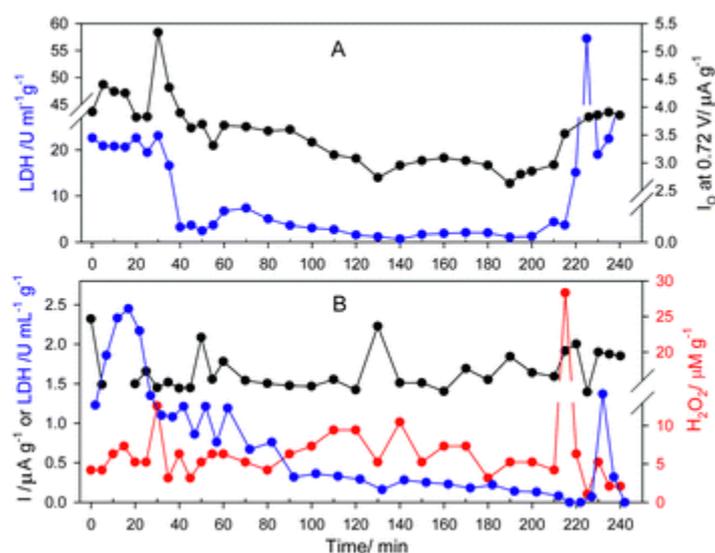


Fig. 5 Comparison of LDH, H₂O₂ and total ROS by EC in human sample perfusion. (A) Release of LDH (blue circles) and ROS by cyclic voltammetry (filled circles). In situ electrode cleaning by DPV, scanning from -0.5 V to 1.5 V before the 25th, 55th, 140th and 190th min. (B) LDH (blue circles), H₂O₂ (red circles) from Amplex Red assay and ROS by cyclic voltammetry (filled circles) with perfusion. The electrode was polished before each scan. Perfusion with 95% O₂-5% CO₂-KHGB with E_s until the 200th min then electrical stimulation was turned off and the sample was incubated with Triton X100-KHGB in (A), or 95% N₂-5% CO₂-KHGB in (B). Stimulation pulse: 4 V cm⁻¹, 1.5 Hz; EC measurements were carried out with the pulse off. (Representative of three independent experiments.)

Fig. 5B shows the comparison of LDH, H₂O₂ release with total ROS by electrochemical measurement in a polishing mode. During perfusion, cyclic voltammetry and SWV measurements took 1.5 min, while it took 2 min to collect sufficient effluents for H₂O₂ assay and another 2 min for LDH analysis. Taking these small differences into account, the LDH release matched the H₂O₂ levels, except between 100 and 160 min where there was a raised level of H₂O₂, with only minor fluctuations in LDH. However, the I₀ in cyclic voltammetry and SWV agreed well with the H₂O₂ assay results in the main, especially at 130 min where LDH did not detect damage (see ESI 4, Fig. S3† for SWV results).

The [H₂O₂] at 220 min was determined to be 1.18 μ M from the off-chip biochemical assay and 3 μ M by electrochemical means after calibration with standard H₂O₂ in the flow system (ESI 5, Fig. S4†), indicating that the total ROS included H₂O₂ plus additional ROS. As stated above it has been reported that H₂O₂ was released from rat heart tissue at a rate of 50 nmol g⁻¹ min⁻¹ two minutes after an ischaemia/reperfusion incident.³⁰ Given the chamber size of the device used in this work was 0.4 mL, with a tissue weight of 0.03 g, these parameters would give an H₂O₂ concentration of approximately 3.75 μ M min⁻¹ for our tissue. In contrast, a release rate of 3×10^{-18} mol s⁻¹ per cell for H₂O₂ by human neutrophils has been reported.³⁴ Assuming an average rat or human cardiomyocyte from the ventricle has a volume $30\,000$ μ m³ (this varies considerably with age and sex)³⁵ and tissue biopsy dimensions of $2 \times 4 \times 4$ mm³ would give a calculation of 0.48 μ M min⁻¹ for H₂O₂. These values do not take into account differences in cell type, nature of the cell sample, i.e. in the tissue a restricted number of cells are initially exposed to the Triton X100, or stimulation method, however the production rates are of a similar scale. Interestingly the H₂O₂ release occurred prior to LDH, indicating that oxidative damage preceded membrane damage and LDH release.

It was found that 95% N₂-5% CO₂ was a good alternative to Triton X100 for inducing cell damage, due to the gas having less effect on the background current in cyclic voltammetry. It induced a similar release of LDH 10 min earlier than Triton X100 did (the 240th min in Fig. 3B and the 230th min in Fig. 5B). However, the LDH rise was smaller than that caused by the detergent treatment. This is most likely due to Triton acting directly on the cell membranes rather than an indirect effect associated with a lack of oxygen. It is hypothesised that longer periods of perfusion with the 95% N₂-5% CO₂ media would result in a similar level of cell rupture and LDH release.

Conclusions

A microfluidic device has been developed for heart tissue perfusion with real-time electrochemical monitoring of ROS release. Having optimized the perfusion and stimulation conditions, it is possible to maintain viable tissue biopsies from rat and human tissue for up to 300 min, a typical time frame for current in vitro models. In situ, real-time electrochemical measurement of total ROS was achieved, matching the release of LDH determined off-chip. Both cyclic voltammetry and square wave voltammetry have been utilised to monitor ROS levels and the concentrations detected have been verified by biochemical assay of H₂O₂. In summary, this study describes a novel method to monitor continuously the change of ROS and other factors in heart tissue biopsies, and will facilitate investigations of normal and pathological cardiac functions ex vivo, whilst mimicking the in vivo environment.

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