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A Robust Microfluidic *in vitro* Cell Perifusion System

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***Abstract*— We present here a *robust* microfluidic cell perifusion device for *in vitro* primary tissue cell secretion studies. This system increases the sample concentration to perifusion volume ratio by an order of magnitude compared with standard multi-well plate static incubation assays. Further, this device achieves physiologically relevant flow rates, pressures, and temperature. It has been manufactured with typical machining facilities, principally drilling and milling. No specialist clean room equipment is required to replicate it. We show its capability here with hormone perifusion experiments on primary pancreatic tissue from mice. This device can increase cell secretion concentrations by up to a factor of 20, allowing for the first time the direct measurement of islet glucagon using mass spectrometry.**

I. Introduction

Microfluidic perifusion systems can improve temporal and spatial control over the environment around cells and tissue placed within them. The chemical and physical effects that cells experience *in vivo* can be accurately mimicked and modeled *in vitro* using these systems, which can increase accuracy of measurements [1]. Concurrent with the development of microfluidic systems is the drive for artificial organ technologies, such as the artificial pancreas for type 1 diabetic treatment [2 - 4].

Glucagon and insulin are hormones released from the alpha and beta cells of pancreatic islets of Langerhans that regulate blood glucose levels. The role of glucagon in the prevention of low blood sugar (hypoglycemia) is prevalent in diabetic control research [5, 6]. However, the measurement and analytical modeling of glucagon is complicated by: (a) the scarcity of primary islets of Langerhans tissue for *in vitro* study [7] (b) low alpha cell numbers which vary between species from 15-30% of islet the total cell population [8, 9] (c) the release of glucagon in concentrations, that are typically three orders of magnitude less than insulin [10] (d) the pulsatile nature of glucagon release, which oscillates over a period of 7 to 14 minutes [11, 12] and (e) species variations between animal models and humans of the signaling pathways and mechanisms of action [13].

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Multi-well plate static incubation assays, are practicably simple and cost-efficient. In comparison, perifusion studies are technically complex, however they can mimic microvasculature of the body more accurately, which is particularly important for islet studies [14, 15]. Alpha and beta cell secretions are dependent on feedbacks from glucose, insulin, and glucagon, amongst myriad internal and external affects [13]. By replacing the extracellular environment over time, perifusion flow rates can influence the level of interaction and feedback experienced by cells [14, 15].

Published islet perifusion techniques include filter chamber systems, BioGel bead matrix columns, and parallel perifusion column devices [17 – 20]. These systems have been successful in elucidating the underlying biology of insulin secretion in beta cells. There are few reports, however, of glucagon perifusion investigations, and those that exist do not all achieve physiologically relevant pressures (<5mmHg), or perifusion volumes that allow high-resolution time sampling [11, 19, 21]. Recently, microfluidic systems have been used to study islets [22 – 25]. Common to these systems are the need for clean-room fabrication facilities, which can introduce supply limitations.

II. Our Approach

We have developed a microfluidic cell perifusion system that measures glucagon secretion using a design that is simple to manufacture, can be re-used and maintained in a typical biological laboratory, and in which all parts are easily accessible from laboratory supply companies. The system increases glucagon sample concentrations beyond two orders of magnitude greater than the minimum detection assay sensitivity limit (radioimmunoassay, RIA). This allows us to sample 24 individual islets at two minute intervals, whist achieving chamber pressures below 5mmHg and flow rates between 10-20µl /min [28]. The device has increased the available quantities of glucagon for assay to the extent that we were able to investigate the use of mass spectrometry (MS) to measure glucagon secretion.

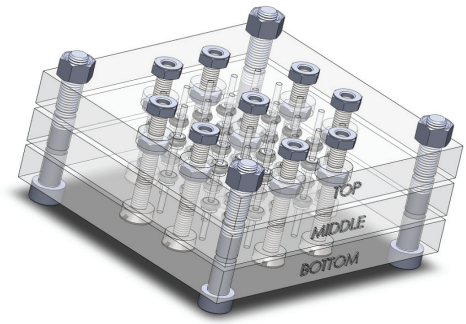

Figure1. Drawing of the perifusion device, showing the three chamber layers held by bolts.

As an alternative analytical tool to Radioimmunoassay (RIA) or Enzyme-linked immunosorbent assay (ELISA), tandem quadropole mass spectrometry can provide highly specific semi-quantitative or quantitative data of multiple analytes within the same sampling volume. Therefore visualization of more than one analyte in a selected sample is possible providing novel information in a single run and limiting the need for repetitive measurements.

III. Methods

A. Device Assembly

The device is a three-tiered system that when assembled forms a chamber, into which cells may be pipetted. Figure 1 shows a drawing of the assembled device; the central layer forms the cell chamber. The device Top layer holds inlet tubing in place and directs reagent flow into the central chamber holding the islets. The Middle and Bottom layers make the cell chamber with porous membranes held between them. Each chamber volume is 12 microliters and the device has 24 chambers. Outlet tubing attached to the Bottom layer allows secretants from the chamber to be collected into eppendorfs.

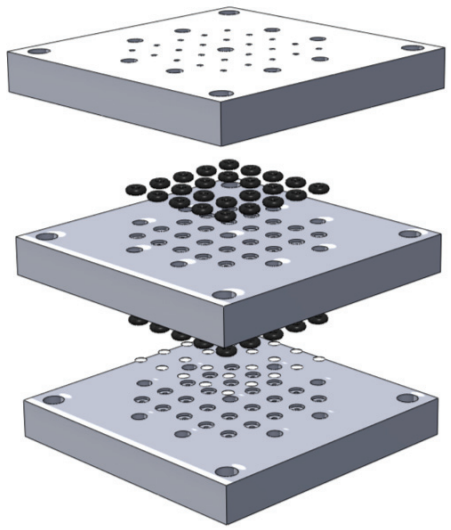

Figure 2. Exploded view of the perifusion device, showing the three chamber layers. Above the bottom layer are the porous membranes, which hold the cells in place. O-rings form a seal between the device chambers. The top and bottom devices hold perfusion inlet and outlet tubes in place.

Cells are loaded vertically into short 1mm inner diameter (ID) inlet tubing using a pipettor with a tip that has a <1mm outer diameter (OD). The inlet tubing is connected upstream to a syringe containing reagent solution. After islet loading, the device and tubing are placed into a water bath at 37°C.

B. Principal Considerations for in vitro cell studies

a) Temperature- Mammalian cell metabolism is sensitive to ambient temperature fluctuations from 37°C [15]. Cell chamber temperature was controlled by submerging inlet tubing such that the perfusate reaches 37°C before it arrives at the chamber. The required submerged lengths are dependent on perfusion flow rate and the external buffer temperature.

b) Materials- The device structure is machined from Polymethylmethacrylate (PMMA), the inlet and outlet tubes are polytetrafluoroethylene (PTFE), the membranes are polycarbonate or Nylon.

c) Membranes- Islet diameters range from approximately 50 µm to 200 µm. 30 µm and 40 µm polycarbonate and nylon membranes have been verified as suitable.

d) Pressure- Chamber pressure was recorded using a Millar gauge (PCU-2000) using flow rates between 10 µl min^{-1} (< 1mmHg) and 1000µl min^{-1} (< 20mmHg).

IV. Experimental Procedure

Islet isolation- Female CD1 mice (8-24 weeks old) were sacrificed by cervical dislocation, in accordance with the UK Home Office Animals Scientific Procedures Act, 1986. The pancreas (5.0 ml per mouse) was inflated via the pancreatic duct with collagenase (1.0 mg ml^{-1}). The distended pancreata were then incubated in a shaking water bath (37° C for 10 min) and recovered using a Histopaque gradient (3ml 1.119g l^{-1}, 3 ml 1.083g l^{-1}, 3 ml 1.077 g l^{-1}). Islets were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and antibiotics and incubated in a humidified atmosphere at 37° C with 5% CO_2. They were cultured between 4-7 days before assay [26].

Cell solutions- D-Glucose was dissolved in Krebs' Ringer bicarbonate HEPES solution containing (mmol/l: 130 NaCl, 3.6 KCl, 1.5 $CaCl_2$,, 0.5 $MgSO_4$, 0.5 KH_2PO_4, 2 $NaHCO_3$, 10 HEPES pH 7.4 with NaOH. The solution was supplemented with 0.1% (wt/vol) Bovine Serum Albumin and bubbled with 95% O_2, 5% CO_2 for 30 mins.

Perifusion cell assay- Between four to twenty islets per chamber were loaded into the device. Glucose solution (1,3 or 17 mM) was pumped at a flow rate of 20 µl min^{-1}. Once all islets were loaded, the device was submerged into the water bath and left for 30-60 mins for islet acclimatisation. Cell secretion at high glucose was collected for 10 minutes, and then buffer was switched to low glucose. Samples were collected every 2 mins for 1 hour and placed immediately on ice for analysis by glucagon Radioimmunoassay. As a positive control, 14 mM Arginine hydrochloride solution was perfused at the end of the glucose collections to maximally stimulate glucagon secretion (Data not shown).

LC-MS and MS/MS Analysis-100 islets were placed in the perifusion system kept at 37° C for 1 hour at 0.1 mM glucose. Islet solutions were prepared for analysis using the procedures described above. The sample solution was acidified with 0.1% Formic acid ready for analysis. Preliminary semi-quantitative Multiple Reaction Monitoring data were obtained using a XevoTQ-S instrument on-line to a Waters Aquity LC with a C18 microbore column.

V. Results

Glucagon hormone secretion in pancreatic islets cells is maximally stimulated by low glucose concentrations (0-3mM) [27]. The islet glucagon response was recorded every

2 mins at 1, 3 and 17mM glucose concentrations using the microfluidic perifusion device.

A. Effect of large glucose step concentration on islet glucagon secretion. Decreasing glucose concentration from 17 mM to 1 mM in Figure 3 resulted in a glucagon peak 10 fold greater than glucagon release before the switch..

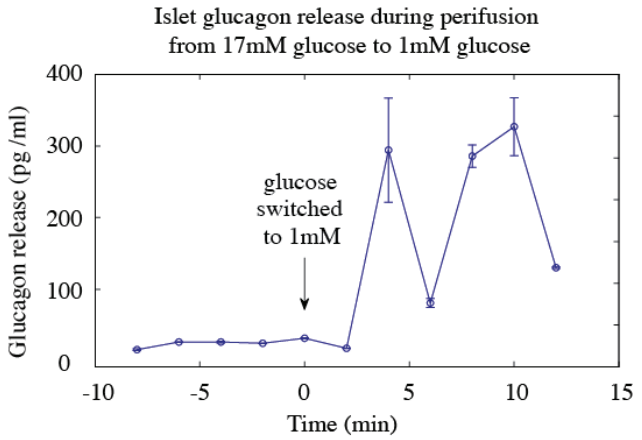

Figure 3: Glucagon secretion at 17mM glucose followed by 1mM glucose perifusion.

B. Effect of small glucose step concentration on islet glucagon secretion. In Figure 4 glucagon release from islets tested with a 3 mM glucose solution was ≈3 fold less when the glucose was switched to 1mM.

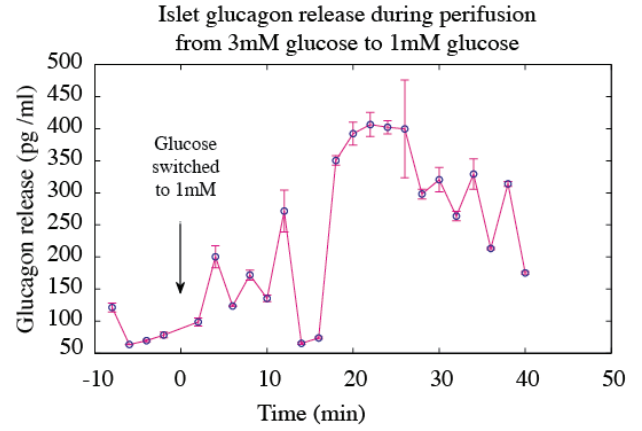

Figure 4: Glucagon secretion at 3mM glucose followed by 1mM low glucose perifusion

C. Glucagon release measured in a static incubation assay and a perifusion assay using the microfluidic device.

Glucagon concentrations collected from a static incubation technique relative to concentrations recorded from the perifusion device displayed in Table 1 show that the maximal glucagon sample concentration was increased by 20 fold compared to the static incubation concentrations, when glucose was reduced from 17mM to 1mM.

Islets No.	Glucose conc. step (mM)	Normalized Glucagon Conc.(pg/ml)	Glucagon Conc./ Islet
12	**10 and 0.1**	**22.8 ± 2.7**	**1.9**
50	**10 and 1.0**	**540.2 ± 17.8**	**25.1**
12	**10 and 1.0**	**20.1 ± 3.3**	**1.7**
40	**17 and 1.0**	**1347.9 ± 51.4**	**33.7**

Table 1: Islet glucagon concentration recorded from the microfluidic perifusion device and a multi-well plate static incubation assay under different physiological conditions

D. In vitro glucagon Multiple Reaction Monitoring (MRM)

Data from an islet-derived sample that was analysed directly, i.e. not desalted prior to MS analysis, is shown in Figure 5. 10µl from 5ml of collected islet secretions was loaded directly onto the Xevo LC-TQ-S, equivalent to the secretion from 0.2 islets (inferred from 100 islets in 5mls). This was sufficient to produce the data shown in Figure 5 below. Future experiments will include full quantification of our islet glucagon samples by co-running synthetic internal standards with the islet samples for quantitative MRM data.

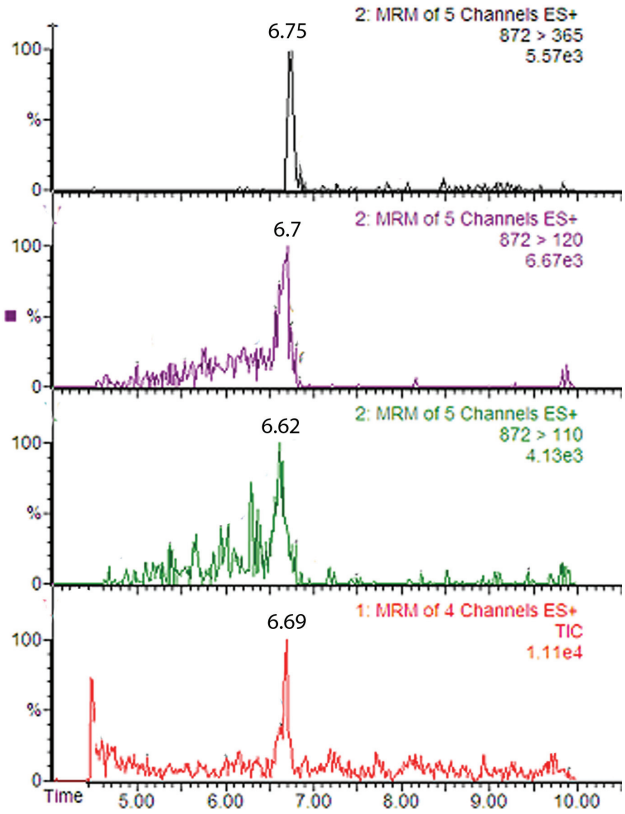

Figure 5: Shows the MRM islet glucagon data looking at the 872(4+) multiple charge state precursor ion observed eluting at 6.5mins. The peptide fragment ion transitions displayed are the N-terminal Histidine a_1 ion m/z 110 and the C-terminal daughter $365y_3$, and 120 y_1 ions

VI. Discussion

The data presented here in Figures 3 and 4 and described in Table 1 validate that this device provides an environment in which islets function with expected physiological behavior [27]. When glucose concentration is decreased from ≥10mM to ≤1mM, data from the perifusion device

displayed a glucagon sample increment between 13 and 20 fold more than typical concentrations recorded from static incubation assays under similar conditions.

Glucagon secretion rates calculated from the experiments described in Table 1 were 1.67pg/islet/min and 2.3pg/islet/min. In comparison, glucagon perifusion data from Kalkhoff *et al.* measured a rate of 0.6-0.8pg/islet/min at low glucose 2.8mM, which is favorably comparable to the data presented here. However, the perifusion set-up described by Kalkhoff et al required the use of 90-100 islets and implemented a flow rate of 0.9ml/min, which is approximately double the islet number required here, and over 400 times the physiological flow rates [19, 28].

The detection of islet glucagon using mass spectrometry illustrates the capacity of the device to optimize measurable sample glucagon, providing the researcher with greater opportunity to explore other areas previously limited by sensitivity, including the quantitation of multiple analytes.

Careful design and testing for robustness including Human Factors testing analysis and design feedback with assembly testing from trained and non-trained personelle has ensured that the device can be constructed in any wet lab. It may be autoclaved so it can be recycled without degradation in use. Further, the ready availability of parts required to make and maintain this device ensures that damaged or lost components can be replaced.

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