FULL PAPER
THE ANALYST
www.rsc.org/analyst

Real-time determination of glucose consumption by live cells using a lab-on-valve system with an integrated microbioreactor

Craig M. Schulz* and Jaromir Ruzicka

University of Washington, Department of Chemistry, PO Box 351700, Seattle, WA 98195, USA. E-mail: cschulz@u.washington.edu; Fax: 206-685-8665; Tel: 206-543-3798

Received 15th July 2002, Accepted 14th August 2002
First published as an Advance Article on the web 2nd September 2002

This paper describes a microquantitative method for glucose determination *in situ* of living cells in real-time. In this novel technique adherent cells are cultured onto microcarrier beads and packed into a renewable microcolumn within a microsequential injection lab-on-valve system (μSI-LOV). Glucose sensing is performed through the use of a two-step, NAD-linked enzymatic process. The course of the assay is monitored in real-time, by absorbance of NADH at 340 nm. The microsequential assay based on plug/nozzle design has a linear dynamic range for glucose of 0.1 to 5.6 mM. The design of the (μSI-LOV) system allows the assay to be carried out using only 40 μL of the enzyme reagent and 3 μL of sample. The technique was tested on a murine hepatocyte cell line (TABX2S) adhered to Cytopore beads. Rapid cellular glucose consumption, in this technique, is facilitated by a high cell density, which allows a large number of cells (10^4–10^5) to be retained in a very small volume (3 μL). In turn, this cell density results in the rapid depletion of glucose from the cell medium over short time periods (<2 min). In conjunction with the assay development, the plug/nozzle design and its ramifications on mixing in general are presented and discussed.

Introduction

Glucose is an essential component for a mammalian cell culture, being used by cells as a major source of energy and carbon.[1] The ability to determine the rate at which cells are consuming glucose can be used to assess the overall health and metabolic state of the cell culture. For example, glucose consumption can be used to ascertain the effects of a hypoxic event in cells. During ischemia tissues are deprived of oxygen which results in the rapid production of lactate with an increase in glucose consumption. The ability to measure glucose consumption prior to, during, and after an ischemic event would allow for an assessment to be made as to how the cells are generating energy during each of these phases. A comparison could also be made between the pre- and post-ischemic glucose consumption rate to determine if there is a change in metabolic programming. Such a change has been witnessed in adult cardiac myocytes.[2,3] Prior to an ischemic event, the myocytes generate a large portion of their energy by fatty acid metabolism, subsequent to an ischmeic event the cells derive most of their energy from glucose metabolism. Fatty acid metabolism consumes more oxygen than does glucose metabolism, therefore the later is more suited to the low oxygen environment encountered during ischemia.

There are numerous techniques for quantitating glucose in biological matrices,[4,5] some of the most common techniques include, optical biosensors,[6] immobilized enzyme electrodes[7] and radiometric assays.[8] However, none of these techniques allow for the probing of metabolism in cellular tissues in a simple automated fashion. Furthermore, optical sensors and electrodes are difficult to sterilize and are susceptible to protein fouling[9,10] and must be periodically reconditioned. Radiometric assay is a very sensitive technique for monitoring glucose consumption, but requires the use of hazardous radiolabels. The novel μSI-LOV system allows metabolic fluxes to be probed in an automated fashion on a renewable microcolumn of cellular tissue, without the use of radiolabels.

This paper describes the design, characterization and implementation of a novel technique for determining the components of cellular metabolism, as shown on glucose consumption. The method is based upon microsequential injection within the lab-on-valve platform (μSI-LOV).[11] This work uses μSI-LOV and the principles of enzymatic analysis for glucose determination in buffer flowed through a renewable microcolumn of bead adherent cells. Previously, the LOV platform has been successfully used to monitor a yeast fermentation by enzymatic analysis,[12] and bead adherent cells have been used in a sequential injection microscopy platform to measure calcium fluxes,[13] oxygen consumption[14] and extracellular pH.[15] The design and principle of this new application of μSI-LOV are shown in Fig. 1. For this application, cells grown on microcarrier beads are packed and perfused in a microcolumn within a microbioreactor, while the perfusate is monitored for glucose by enzymatic analysis in the flow cell by optical fibers. The essential component of this design is a plug that fulfills two functions: retaining the beads upstream, yet in close proximity to the flow cell, and second, serving as a nozzle through which the assayed stream is forced into stagnant reagent held in the flow cell, causing instant mixing of sample and reagent. The result is a novel process that allows for a rapid real-time assay of glucose consumption by live cells within a microbioreactor integrated into the LOV design.

For this project, Infinity™ glucose reagent (Sigma Chemical, St. Louis, MO) was selected as a well characterized assay for glucose detection.[16] The assay is based upon a two-step enzymatic process, beginning with the phosphorylation of glucose by hexokinase and the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP):

$$\text{glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP}$$

Next, glucose-6-phosphate (G-6-P) is oxidized to 6-phosphogluconate (6-PG) with a coupled reduction of NAD^+ to NADH. NAD^+ and NADH are abbreviations for nicotinamide adenine

dinucleotide in the oxidized and reduced forms respectively. This reaction is catalyzed by glucose-6-phosphate dehydrogenase and is expressed as follows:

$$\text{G-6-P} + \text{NAD}^+ \rightarrow \text{6-PG} + \text{NADH} + \text{H}^+$$

As the reaction proceeds, NAD^+ is reduced to NADH, which has absorbance at 340 nm. Therefore, the progress of the reaction can be monitored by measuring absorbance at 340 nm.

Experimental

Instrumentation

Apparatus. An FIAlab 3000 (FIAlab Instruments, Medina, WA) was used for this study. Included in the instrument is a high precision bi-directional syringe pump with a 1.0 mL syringe, a two-way valve, and a 6-position lab-on-valve (LOV).

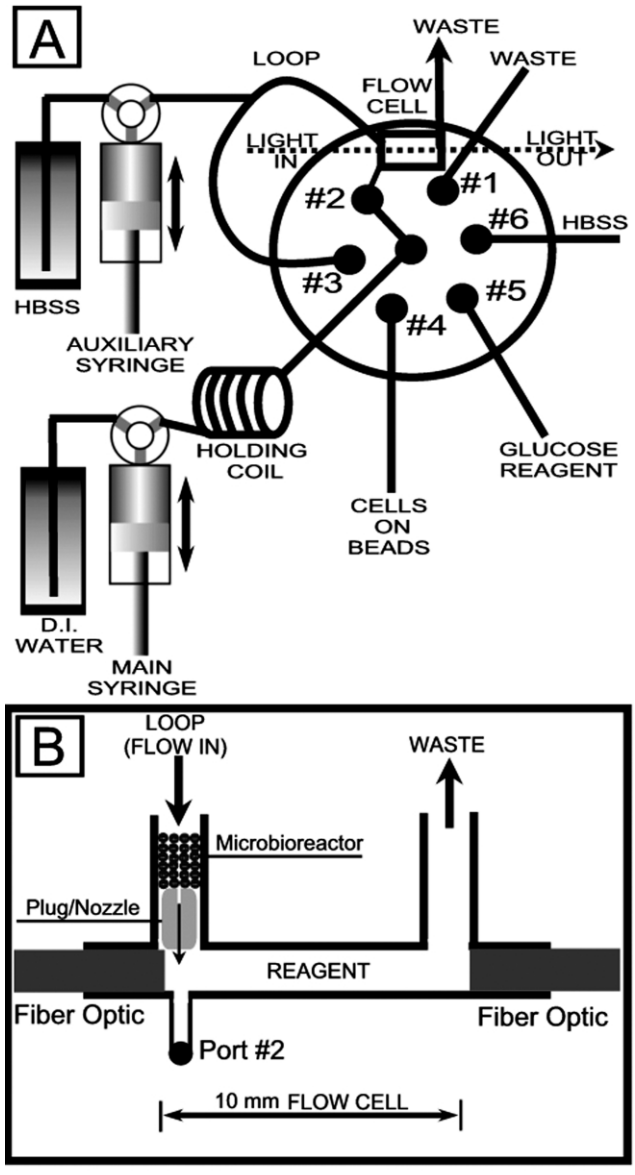

Fig. 1 μSI-LOV system. (A) Schematic depiction of the μSI-LOV system. (B) Principle: cells grown on microcarrier beads are retained by the plug, furnished with a nozzle (175 μm id), through which the analyzed stream enters stagnant reagent at a high linear flow velocity.

A loop was made by connecting port #3 of the LOV to one of the flow cell ports. Additionally, an auxiliary Cavro XL 3000 syringe pump with a 250 μL syringe (Cavro Scientific Instruments Inc., Sunnyvale, CA) was connected to the loop *via* a solution tee (Fig. 1A). The system was plumbed with 1.59 mm od (outer diameter) PEEK and stainless steel tubing (Upchurch Scientific, USA). The bead retention plug/nozzle (Fig. 1B) was made from a 2 mm length of 0.178 mm id (inner diameter) PEEK tubing. The retention plug/nozzle was inserted into a channel of the LOV, and was kept from entering the flow cell by the end of a fiber optic. The void volume of the plug was approximately 0.5 μL. FIAlab software version 5.0 (FIAlab Instruments, Medina, WA) was used to control all of the system components and was also used for data collection and analysis. The flow cell was illuminated by a long wave UV pencil light (Spectronics Corp., Westbury, NY) coupled to a 600 μm UV fiber optic. Absorbance measurements were accomplished *via* a 600 μm UV fiber optic connection to an Ocean Optics SD-2000 CCD spectrophotometer (Ocean Optics, Dunedin, FL) containing a #1 (UV sensitive) grating. Absorbance was monitored at 340 nm at a frequency of 1.5 Hz with 500 ms of integration. The fiber optics were spaced 10 mm apart, resulting in a flow cell volume of 22.7 μL. The entire apparatus was placed inside of an Imperial III incubator (Lab-Line, Barnstead Int., Dubuque, IA) to provide precise temperature control over all of the instrument components, as well as to maintain a physiological temperature (37 °C) for all studies. An expanded view of the LOV flow cell is represented in Fig. 1B with a photograph of the LOV in Fig. 2A.

LOV design. This work represents the first assay of live cells carried out within the lab-on-valve (LOV) platform. For cellular studies, it is not desirable to have the reagent come into contact with the cells as their function may be perturbed by changes in pH, osmotic pressure or toxicity of reagents. Therefore, the present design allows for the first time, separation of the microbioreactor volume from the detection volume of the flow cell (Fig. 1B; Note: The term microbioreactor refers to the volume in which the microcolumn is packed and the cellular reactions are allowed to proceed, which should not be confused with the enzyme reaction that occurs downstream in the flow cell). A loop was fashioned to connect port #3 and the flow cell (Fig.1A). This configuration allows for an aliquot of beads to be delivered through port #3 and captured in a microcolumn behind the bead retention plug/nozzle directly upstream from the flow cell (Fig. 1B). Once a microcolumn is packed it can be perfused by either of the syringe pumps, through the loop, with the resulting perfusate entering the flow cell through the plug/nozzle. Inside the flow cell, the analyte is mixed with reagent for detection. Thus, in this novel configuration, a microcolumn of cells can be monitored while the reagents do not come into contact with the cells, and a very small sample surrounding the cells (3 μL) has to travel through only a short nozzle (0.5 μL) to be mixed with an excess of enzyme in the 22.7 μL volume of the flow cell.

Materials and reagents. Infinity™ glucose reagent, and glucose standard were obtained from Sigma Chemical (St. Louis, MO). All standards and reagents were prepared fresh daily using ultrapure water made by a Nanopure system (Barnstead Thermolyne Corp., Dubuque, IA). The glucose reagent was at a final concentration of >1500 U L^{-1} hexokinase, >3200 U L^{-1} glucose-6-phosphate dehydrogenase, 2.1 mM ATP and 2.5 mM NAD^+. The glucose reagent had a final pH of 7.5. Cytopore beads were obtained from Amersham Pharmacia Biotech, Uppsala, Sweden. Hank's Balanced Salt solution (HBSS) was obtained from Gibco (Gibco BRL, Grand Island, NY). The HBSS was fortified with

calcium chloride (Sigma Chemical, St. Louis, MO) to a final concentration of 1.8 mM.

Cell culture. The mouse hepatocyte cell line, TABX2S, was grown in complete medium consisting of Dulbecco's modified Eagle's medium/Ham's F12 medium with 5 mg mL^{-1} insulin, 5 mg mL^{-1} transferrin, 5 ng mL^{-1} selenium, 100 nM dexamethasone and 10 mM nicotinamide. These cells were grown to approximately 80% confluence on a 150 cm^2 polystyrene cell culture flask. Cells from the flask were trypsinized to release them from the surface, washed in fresh medium, and used to inoculate a 10 mL culture tube containing 20 mg dry weight of Cytopore microcarrier beads. The beads were prepared according to the manufacturer's instructions. Briefly, 20 mg of dry beads were hydrated overnight in 1 mL Hank's balanced salt solution (HBSS). The beads were sterilized by autoclaving at 121 °C for 30 min. An aliquot of beads corresponding to 20 mg dry weight was equilibrated into cell culture medium by washing several times. The bead medium slurry was warmed to 37 °C prior to inoculation with the cells. After inoculation, the cells and beads were gently stirred and settled several times in a 15 mL Falcon tube. The cells were cultured at 37 °C with 5% CO_2. Prior to experimentation, the cells were washed several times with HBSS, this step was used to remove cellular debris and to wash off the phenol red component of the media. The final dilution was, 1 mL of bead slurry in 10 mL of HBSS.

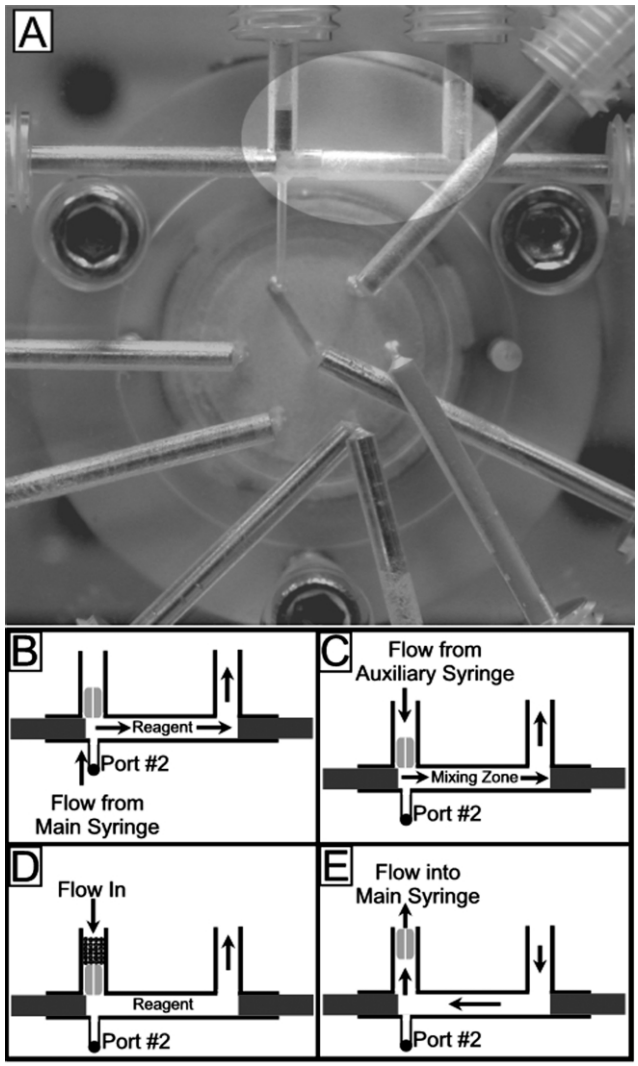

Fig. 2 LOV flow schemes. (A) Highlighted area of the LOV demonstrates the flow cell. (B) Introduction of enzyme into the flow cell. (C) Sample injection and mixing. (D) Column packing behind the bead retention plug/nozzle (The size of the beads is exaggerated for clarity). (E) Removal of a packed microcolumn from the microbioreactor.

Results and discussion

Glucose assay development

A glucose assay, in the absence of beads, is initiated by filling the auxiliary syringe (250 μL) with glucose standard. The loop is then filled with the same standard, by dispensing several syringe volumes through the loop. Next, the flow cell (port #2) is flushed, using the main syringe pump (1.0 mL), with deionized water. The flow cell is then loaded with enzyme reagent by aspirating 40 μL of the glucose reagent (port #5) into the holding coil using the main syringe pump. The valve is then switched to the flow cell position (port #2). Once the valve is switched, the enzyme is dispensed into the flow cell using the main syringe pump, which is then placed in stop flow mode (Fig. 2B). Next, 3 μL of glucose standard is dispensed into the flow cell through the plug/nozzle using the auxiliary syringe pump, which is then placed in stop flow mode (Fig. 2C). The spectrometer is zeroed at the point of mixing, and signal is acquired for 80 s, during this time period, both pumps are in stop flow mode.

The protocols described in this section were carried out under software control, which allows the entire process to be automated and performed in a precise and reproducible manner. Automation also increases the overall throughput of the technique.

Assay optimization and calibration

The protocol for the glucose detection was optimized by varying the flow rate at which the sample is pushed through the retention plug. (Note: in the absence of the plug, unsatisfactory mixing was obtained at all of the employed flow rates). The resulting reaction rate curves monitored over an 80 s time period for varied delivery rates of 3 μL of 1 mM glucose are shown in Fig. 3 (each run was done in triplicate). At slow flow rates (< 10 μL s^{-1}) the introduced sample mixes poorly with the reagent, and the reaction does not begin to proceed until the layers mix by diffusion. At higher flow rates (> 10 μL s^{-1}) the reaction proceeds as expected when the two components are fully mixed, with the steepest portion of the reaction curve observed during the initial portion of the reaction. The maximum reaction rate is obtained at 40 μL s^{-1}, however it has been noted that the microcarrier beads begin to squeeze past the retention plug/nozzle at flow rates higher than 30 μL s^{-1}. It was decided to use 30 μL s^{-1} as the optimal flow rate for this technique, at this flow rate adequate mixing is achieved and the bead microcolumn is not displaced from the microbioreactor. It is speculated that the improvement in mixing observed at higher flow rates is due to increasing turbulent flow caused by the presence of the plug in the flow channel. In order to verify this assumption, the Reynolds number (*Re*) was used to estimate whether fluid flow will be laminar or turbulent at steady state flow in a straight channel. The calculated Reynolds number, as fluid flows through the bead retention plug/nozzle and into the flow cell (Fig. 2C) at a flow rate of 30 μL s^{-1}, is approximately 1.5–2.0. Since the cutoff for turbulent flow is at an *Re* of approximately 2000, it is safe to assume that the flow within the plug is laminar at the flow rates used. However, since sufficient mixing occurs at flow rates of > 10 μL s^{-1}, as is shown in Fig. 3, another explanation is postulated. The mixing of analyte (glucose) with reagent (enzyme) occurs as the stream from the microbioreactor (Fig. 1B and Fig. 2C) enters, at high velocity, the stagnant solution held within the flow cell. Since, in the absence of the plug/nozzle, mixing is unsatisfactory, and since in the presence

of the plug/nozzle, the mixing occurs at flow rates above 10 μL s^{-1}, corresponding to a mean linear flow rate of 8 cm s^{-1}, it is obvious that the mixing is achieved when the exit velocity from the plug/nozzle exceeds a certain linear flow rate (Fig. 4). This is confirmed by the visual observation captured by a video camera at 30 frames per second shown in Fig. 5, where 3 μL of 1 mM BTB was injected through the plug/nozzle at a flow rate of 30 μL s^{-1}. The movie frames demonstrate that the dye permeates the entire flow cell in less than 1 s, and therefore mixing must be a result of the accelerated sample stream meeting with the stagnant reagent.

Once the optimized flow rate for the glucose assay was determined (30 μL s^{-1}), a second series of experiments was conducted to determine the optimum volume of sample to inject through the plug/nozzle. Fig. 6A demonstrates the reactions of differing volumes of 1 mM glucose delivered at 30 μL s^{-1} into the flow cell. The signal steadily increases with larger volumes of sample, up to a maximum at 10 μL. Delivery of volumes greater than 10 μL results in a decline in the signal because the increasing volume of sample displaces the enzyme solution from the 22.7 μL flow cell, thus limiting the amount of available enzyme. Fig. 6B shows a plot of sample volume *vs*. the ratio of absorbance to volume. Injection of 10 μL of sample results in the largest overall absorbance, but 3 μL results in the largest absorbance to volume ratio. It should be noted that there is not a linear relation between the absorbance and volume delivered due to the fact that enzyme is displaced from the flow cell as sample is introduced. Displacement of the enzyme results in a lower concentration left in the flow cell to mix with the sample. This technique was developed to assay liquid from the interstitial volume of a bead column with cells cultured to the surface. Since this volume is quite small, the most efficient assay of sample is more important than the maximum signal obtained by injecting a large volume. Weighing the evidence for sample volume presented in Fig. 6 and discussed above, 3 μL was decided upon as the best volume to use for this assay.

Using the optimum flow rate (30 μL s^{-1}) and volume of sample (3 μL) determined in the previous experiments, the system was calibrated with injections of differing concentrations of glucose from 0.1 to 5.6 mM (Fig. 7). The assay period was reduced, from 80 s to 10 s. The assay exhibits a linear correlation ($y = 0.07$[glucose] + 5 × 10e^{-3}, $R^2 = 0.999$) between the endpoint signal and glucose concentrations from 0.1 to 5.6 mM (Fig. 7 inset). [Note: for the purpose of this work, endpoint will refer to the signal recorded at the end of the *assay period* (corresponding to the last data point acquired), which should not be confused with the *reaction* endpoint (when

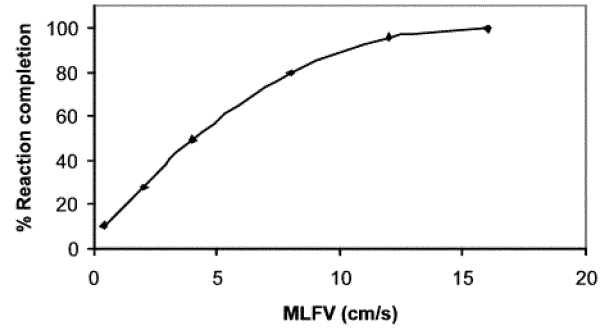

Fig. 4 Percent reaction completion at increasing mean linear flow velocities (MLFV) from 0.4–16 cm s^{-1}.

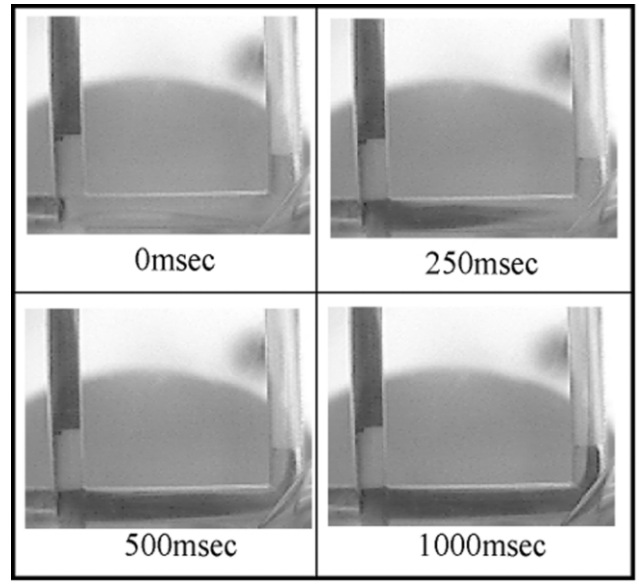

Fig. 5 Time series pictures of a 3 μL injection of 1 mM blue dye (BTB) delivered at 30 μL s^{-1} into the flow cell filled with a colorless solution (borate buffer).

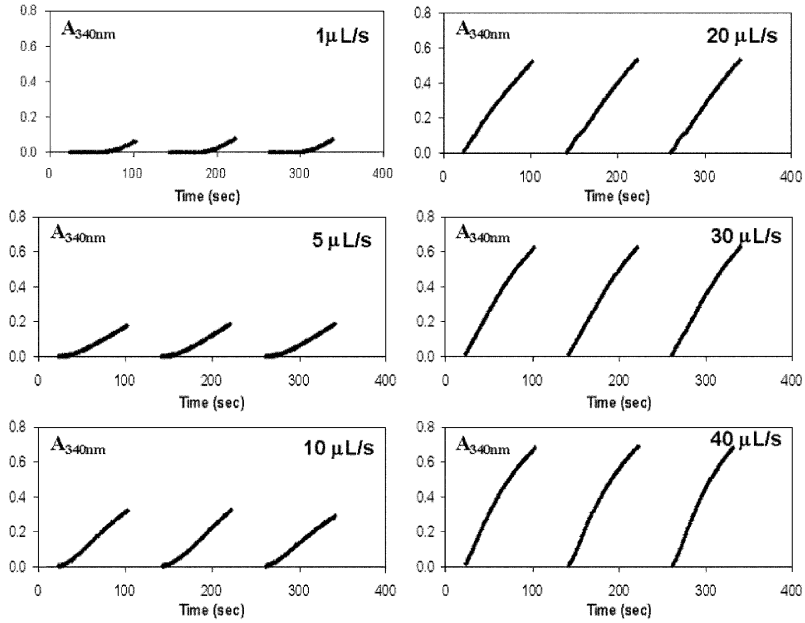

Fig. 3 Reaction rate curves obtained by mixing of 3 μL of 1 mM glucose injected into the flow cell filled with enzyme at flow rates of 1, 5, 10, 20, 30 and 40 μL s^{-1}

products and reactants are at equilibrium)]. The reproducibility of the glucose assay carried out in this manner is approximately ±1% for ten replicate injections, however the day-to-day reproducibility is ±2.5%.

Cell studies

For the cell experiments, a microcolumn of bead adherent cells is packed in the microbioreactor behind the bead retention plug/nozzle (Fig. 2D). For these experiments a microcolumn is packed in the following manner: an aliquot of the bead slurry (port #4) is aspirated into the holding coil using the main syringe pump (1.0 mL). The valve is then switched to the loop position (port #3) and the bead slurry is dispensed through the loop and a microcolumn is packed above the bead retention plug/nozzle in the microbioreactor (Fig. 2D). The microcolumn is then perfused with HBSS using the auxiliary syringe pump (250 μL). The auxiliary pump is then placed in stop flow mode, allowing the cells to consume the glucose from the HBSS contained in the microbioreactor. After a stop flow interval, the HBSS segment is pushed off the cell microcolumn through the plug/nozzle into the flow cell, which is filled with reagent, using the auxiliary syringe pump. At the end of the assay the bead microcolumn is removed from the microbioreactor by aspirating through the loop (port #3) using the main syringe pump. This action drew the beads and the bead retention plug/nozzle backwards toward the holding coil (Fig. 2E). The bead plug/nozzle stops at the fitting interface, while the beads continue moving into the holding coil. The valve is then switched to the waste position (port #1) and the beads are flushed to waste. A new microcolumn of cells-on-beads is then packed in the microbioreactor, and another assay is conducted.

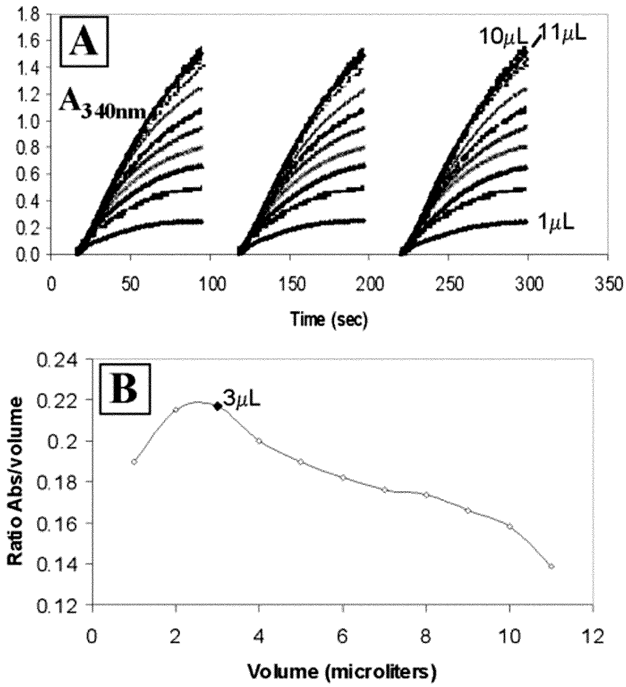

Fig. 6 Increasing sample volumes of 1 mM glucose injected at 30 μL s^{-1} into the flow cell filled with enzyme reagent. (A) Triplicate runs of increasing volumes of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 μL. (B) Plot of injected volume *vs*. ratio of absorbance to volume. Optimal volume of 3 μL is highlighted on the chart.

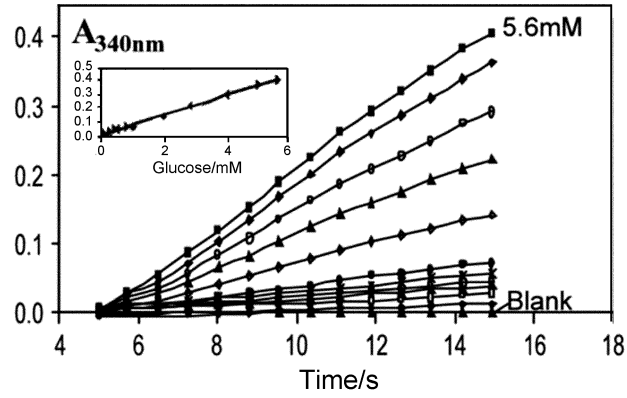

Fig. 7 System calibration. Reaction runs for 0.10, 0.25, 0.40, 0.50, 0.80, 1, 2, 3, 4, 5 and 5.6 mM glucose. 3 μL of glucose was injected at a flow rate of 30 μL s^{-1}, the stopped flow interval was 10 s. Inset: Plot of endpoint signal *vs*. concentration of glucose in mM units.

The results of the initial cell studies are displayed in Fig. 8. The assays were conducted in an identical manner as in the previous studies except the samples were obtained from a cell column (containing 10^4–10^5 cells) filled with 5.6 mM glucose in HBSS buffer, after 0, 60, and 120 s of stopped flow, during which glucose is metabolized by the cells. All of the data presented is the average of three runs. The basal data demonstrates the decrease in the glucose signal in time due to cellular consumption. The azide (N_3^-) data represents the cellular glucose consumption in the presence of 5 mM azide. Azide is a cytochrome C oxidase inhibitor, which results in the fully anaerobic conversion of glucose by the cells. Being that the anaerobic pathway produces less ATP per glucose than does the aerobic pathway, cellular glucose consumption should increase to compensate in the presence of azide. The azide experiments were conducted to verify our initial cellular consumption data. The cell free blank data corresponds to an identical set of runs over a column of blank Cytopore beads.

Conclusion

This paper has described a novel approach to monitor cellular metabolism, whereby cells cultured onto microcarrier beads are retained in a temperature controlled microbioreactor integrated into LOV. This design allows glucose consumption to be monitored in real-time through mixing of the microbioreactor perfusate with glucose reagent inside the flow cell. This work is the first study of live cells held within the LOV while their metabolism is monitored in real-time by optical fibers and UV/VIS spectroscopy. It has been demonstrated that the system provides very rapid response, resulting in short assay periods (10 seconds). Furthermore, the design of the LOV microbioreactor system allows for rapid delivery of glucose to a large number of cells, a task difficult to achieve reproducibly on a monolayer cell culture. The unique fluid handling in μSI mode allows for rapid mixing and detection based on the principles of *programmable flow*, meaning that the flow is stopped, reversed,

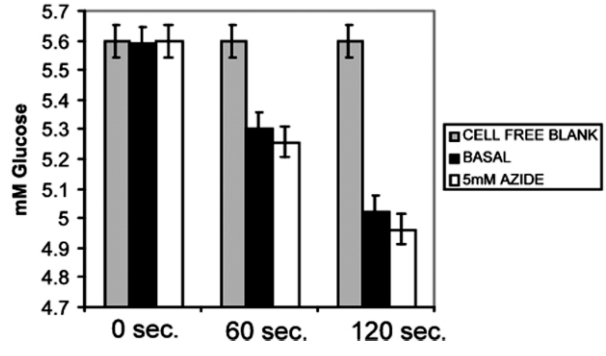

Fig. 8 Initial studies of glucose consumption by live cells. Glucose concentrations were assayed at the end of a 10 second stop flow period. Data were fit to the calibration equation to calculate the glucose concentration. Each bar corresponds to the average of three runs.

or pulsed at different flow velocities. Unlike continuous flow mode, a reaction in the μSI-LOV platform can be initiated by a short accelerated pulse of sample into a stagnant reagent solution held within a flow cell of sufficient volume.

The chief advantages of this approach are, precise handling of the cells and reagents, high reproducibility of measurements, adequate temperature control and the automated renewal of the cell column and reagents. The advantage of the μSI-LOV system over enzyme electrodes and optical sensors, lies in the ability to automatically renew the cell column and the reagent before every measurement. Thus if the cell column becomes fouled or the cells are destroyed, they can be replaced, in an automated fashion, in a matter of a few seconds.

The method described here will also be used for other NAD-linked enzyme assays. Thus, the assay for glucose can be run first, then the reagent will be flushed to waste and the flow cell will be filled with lactate dehydrogenase (LDH). LDH converts lactate to pyruvate and in turn reduces NAD^+ to NADH, thus allowing the reaction rate to be monitored by the accumulation of NADH, identical to the detection scheme for the glucose assay. In this way, simultaneous monitoring of glucose consumption and lactate extrusion from cultured cells, will be accomplished. Comparing the concentration of consumed glucose to the concentration of extruded lactate will allow for an estimate to be made as to the portion of the cells energy that is generated by aerobic and anaerobic metabolism. This technique will also be used to rank the basal metabolism of differing cell lines, as well as, determine the makeup of their respective metabolic regimes (aerobic *vs*. anaerobic). This technique will be especially useful to compare the metabolism of a primary and secondary cell culture. Primary cell cultures tend to be significantly more aerobic than secondary cultures.

This μSI-LOV technique will also be used to ascertain the effects of metabolic enhancing/inhibiting compounds, such as antimycin A. The basal rates of glucose consumption and lactate extrusion can be compared to the rates obtained after dosing with antimycin A. This comparison will then be used to determine the relative effects of the agonist on cellular metabolism. Replicate experiments will be conducted using varied concentrations of the agonist. These experiments will provide a dose response for the compound, from which the effective pharmacological concentration of the agonist can be determined.

Finally, use of nozzle assisted mixing in a programmable flow sequential injection system is a novel approach for more efficient mixing in microfluidic applications. The impact of an accelerated analyte stream aimed through a nozzle at a stationary pool of reagent results in mixing that is more efficient than approaches based on mixing of layers of analyte and reagent formed at laminar flow conditions, where diffusion alone is supposed to provide efficient mixing.[17]

Acknowledgements

This research was funded by a grant from the National Institutes of Health (R01 GM45260-11). We wish to thank our colleagues Michael Manion, PhD, and David Hockenbery, MD (Fred Hutchinson Cancer Research Center, Seattle, WA) for their generous donation of the TAMH cell line and their expertise in cell culture techniques. We would also like to acknowledge Louis Scampavia, PhD (University of Washington, Seattle, WA) for his advice and support.

References

1 L. Stryer, *Biochemistry,* Freeman Press, New York, NY, 4th edn., 1995.
2 P. M. Barger and D. P. Kelly, *Am. J. Med. Sci*., 1999, **318**(1), 36–42.
3 J. Minners, E. J. van den Bos, D. M. Yellon, H. Schwalb, L. H. Opie and M. N. Sack, *Cardiovasc. Res*., 2000, **47**(1), 68–73.
4 D. M. Fraser, *Biosensors In The Body: Continuous In Vivo Monitoring*, Wiley, New York, 1997.
5 S. Aisenberg, *Blood Glucose Monitoring: Methodology and Clinical Application of Continuous In Vivo Glucose Analysis: Proceedings of a Workshop-Conference Held in Freiburg (Germany) 1976*, G. Thieme, Stuttgart, Germany, 1977.
6 *Optical Diagnostics and Sensing of Biological Fluids and Glucose and Cholesterol Monitoring*, ed. A. V. Priezzhev and G. L. Cote, SPIE, Bellingham, WA, 2001.
7 G. Locher, B. Sonnleitner and J. Fiechter, *J. Biotechnol*., 1992, **25**, 23.
8 A. M. Urbano, H. Gillham, X. Groner and K. M. Brindle, *Biochem. J*., 2000, **352**, 921.
9 K. Rebrin, G. M. Steil, W. P. Van Antwerp and J. J. Mastrototaro, *Am. J. Physiol*., 1999, **277**, 561.
10 M. R. Phelps, J. B. Hobbs and D. G. Kilburn, *Biotechnol. Bioeng*., 1995, **46**, 514.
11 J. Ruzicka, *Analyst*, 2000, **125**, 1053–1060.
12 C.-H. Wu, L. Scampavia, J. Ruzicka and B. Zamost, *Analyst*, 2001, **126**, 291.
13 W. L. Connors and J. Ruzicka, *Anal. Biochem*., 1999, **268**, 377–382.
14 I. Lähdesmäki, L. D. Scampavia, C. Beeson and J. Ruzicka, *Anal. Chem*., 1999, **71**(22), 5248–5252.
15 I. Lähdesmäki, J. Ruzicka and A. Ivaska, *Analyst*, 2000, **125**(10), 1889–1895.
16 Sigma Chemical Co., St. Louis, MO, www.sigma.com.
17 F. G. Bessoth, A. J. deMello and A. Manz, *Anal. Commun*., 1999, **36**, 213–215.