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DYNAMIC MONITORING OF GLUCAGON SECRETION FROM LIVING CELLS ON A MICROFLUIDIC CHIP

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Abstract

A rapid microfluidic based capillary electrophoresis immunoassay (CEIA) was developed for on-line monitoring of glucagon secretion from pancreatic islets of Langerhans. In the device, a cell chamber containing living islets was perfused with buffers containing either high or low glucose concentration. Perfusate was continuously sampled by electroosmosis through a separate channel on the chip. The perfusate was mixed on-line with fluorescein isothiocyanate-labeled glucagon (FITC-glucagon) and monoclonal anti-glucagon antibody. To minimize sample dilution, the on-chip mixing ratio of sampled perfusate to reagents was maximized by allowing reagents to only be added by diffusion. Every 6 s the reaction mixture was injected onto a 1.5 cm separation channel where free FITC-glucagon and the FITC-glucagon-antibody complex were separated under an electric field of 700 V cm⁻¹. The immunoassay had a detection limit of 1 nM. Groups of islets were quantitatively monitored for changes in glucagon secretion as the glucose concentration was decreased from 15 to 1 mM in the perfusate revealing a pulse of glucagon secretion during a step change. The highly automated system should be enable studies of the regulation of glucagon and its potential role in diabetes and obesity. The method also further demonstrates the potential of rapid CEIA on microfluidic systems for monitoring cellular function.

Keywords

electrophoresis; immunoassay; cells on chips; microfluidics; glucagon

Introduction

Microfluidics has been increasingly used in combination with cell culture for *in vitro* cell biology studies. Microfluidics allows precise control over cellular environment enabling many experiments not possible with conventional techniques [1–3]. In such experiments, it is often of interest to perform chemical measurements on cells to understand how the cellular environment has affected cell function or biochemistry. Most commonly optical measurements are used to probe cells on chips, but the versatility of cell studies on microfluidic systems can be improved by integrating other chemical measurements. In this work, we demonstrate an approach to measure hormone release from small groups of cells on chips using an electrophoretic immunoassay.

A variety of chemicals are released from cells, including signaling molecules such as hormones or neurotransmitters, trophic and growth factors, and metabolic products. Temporally resolved measurements of cell releasates are important in studying the

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regulation of secretion, determining the effect of drugs (or drug candidates) on cell function, and assessing the health of cells used in transplant or tissue engineering. Measurements of chemical release from cells are typically performed by perfusing cells, collecting perfusate in fractions, and then performing offline analysis by immunoassay or other appropriate methods. This process can be automated and miniaturized in microfluidic systems by direct measurement of release using sensors built into the chip or perfusing cells and mixing the perfusate with assay reagents on-line (if necessary) so that the result of the assay reaction can be detected downstream [4–11]. Demonstrated advantages relative to conventional approaches include: 1) automation; 2) better temporal resolution because of the speed of assays [12]; 3) better sensitivity by reduction of volume and dilution; 4) reduction of amount of cells and reagents required; 5) continuous measurements for up to 24 h [13]; and 6) complex environmental control integrated with measurement [14].

Sensors, enzyme assay, fluorescent assay, and capillary electrophoretic immunoassay (CEIA) have been used for chemical measurement of secretions on chips [4–11]. CEIA is particularly powerful because it can be used for a wide variety of compounds. In this approach, fluorescent antigen (Ag^*) and antibody (Ab) are added to the perfusate containing antigen (Ag) [15]. Electrophoretic separation allows detection of the $\text{Ag}^*\text{-Ab}$ complex and free Ag^* . The relative size of these peaks is quantitatively related to the unlabeled Ag present in the sample. Such systems are well suited for monitoring applications because they can be fast (on-line separations in seconds) and sensitive. Further, they have been shown to be stable enough to allow over 10^4 assays in one monitoring session [13, 16].

Previous work on using CEIA for on-line cell monitoring has been confined to measuring insulin release from single islets of Langerhans [9, 12]. Islets are 75–200 μm diameter spheroid microorgans located in the pancreas that contain 2000–4000 endocrine cells each. Although insulin is the most abundant hormone released from islets, several other hormones such as glucagon are also released from islets to help regulate metabolism. In this work, we have adapted the device to allow for monitoring of glucagon secretion from batches of islets.

Glucagon is a 29-amino acid peptide hormone that acts as the major counter-regulatory signal to insulin [17]. Glucagon is secreted from α -cells in response to low circulating glucose levels to stimulate hepatic glucose production. It is increasingly realized that abnormal regulation of glucagon may play a role in type 2 diabetes and obesity, therefore research into its secretion is growing [18–20]. Study of glucagon secretion is difficult because α -cells comprise only 10–15% of the total islet cell population resulting in relatively low amounts of hormone per islet [21] and small changes in glucagon secretion upon stimulation [22].

In addition to adaptation of the immunoassay to glucagon, we have modified a previously described microfluidic system [12] to implement a novel approach to reagent addition that minimizes dilution of sample. These results provide a novel tool for studying glucagon secretion, validate a new method of mixing reagents that minimizes dilution of sample, and demonstrates the versatility of the microfluidic CEIA approach for cell secretion measurements.

Experimental

Chemicals and Reagents

Cell culture reagents and fluorescein-5-isothiocyanate (FITC) were from Invitrogen (Carlsbad, CA). Tween-20, collagenase type XI, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), glucagon and monoclonal antibody to glucagon (clone K79bB10; $K_a = 10^9 \text{ M}^{-1}$) were from Sigma-Aldrich (St. Louis, MO). All solutions were

made from Milli-Q (Millipore, Bedford, MA) $\geq 18 \text{ M}\Omega \text{ cm}^{-1}$ deionized water. All other chemicals were obtained from Fisher (Hampton, NJ) and were of the highest purity available. Glucagon standard solutions were prepared daily. Stock Ab was maintained in aliquots at -32°C in the manufacturer provided ascites fluid. FITC-glucagon fractions (labeling described below) were stored at -32°C in HPLC buffer. Bulk solutions were filtered using $0.2\text{-}\mu\text{m}$ nylon syringe filters (Fisher). Glucagon or antibody containing solutions were filtered using $0.2\text{-}\mu\text{m}$ inorganic Anotop 10 syringe filters (Whatman, Florham Park, NJ) pre-conditioned with BSA containing solutions.

Reagent buffer consisted of 20 mM NaCl, 50 mM tricine, 1 mM EDTA, pH 7.4, supplemented with 1 mg/mL BSA and 0.1% (w/v) Tween 20. Electrophoresis buffer consisted of 150 mM tricine, 20 mM NaCl at pH 7.4. Balanced salt solution consisted of 125 mM NaCl, 5.9 mM KCl, 1.2 mM MgSO_4 , 2.4 mM CaCl_2 , 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 7.4, and 1 mg/mL BSA.

Preparation of FITC-glucagon

Synthesis of FITC-glucagon was performed under conditions similar to those used previously to obtain singly labeled insulin [23]. The labeling reaction was initiated by addition of 0.1 mL of 3 mM FITC dissolved in acetone to 0.9 mL of 0.3 mM glucagon dissolved in 100 mM phosphate buffer, pH 7.5, while stirring. The reaction proceeded unstirred, in the dark, at room temperature for 24 h. HPLC purification ($100\text{-}\mu\text{L}$ injection loop) was performed using a Grace-Vydac C_4 column ($5\text{-}\mu\text{m}$ d_p , $250 \times 4.6 \text{ mm}$, 214TP54, Columbia, MD) and a linear gradient of 0 – 40 min (20% to 38% B), where A was 0.1% trifluoroacetic acid in deionized water and B was 0.1% trifluoroacetic acid in acetonitrile, while maintaining 1.5 mL/min flow rate. Three major peaks were observed and identified as shown in Electronic Supplementary Material Fig. S1. Free FITC and unlabeled glucagon were identified by comparison of retention time with standards. FITC-labeled glucagon peak was tentatively identified based on its appearance after the reaction and confirmed by CEIA (see results). Minor peaks were due to FITC impurities and present in individual FITC runs. Concentration of FITC-glucagon in purified fractions were determined by UV absorption at 494 nm in pH 8 phosphate buffer based on the molar absorptivity of fluorescein ($\epsilon = 68,000$).

Instrumentation

Fig. 1 illustrates the design of the microfluidic device used in all experiments. Channel dimensions were $6 \mu\text{m}$ deep, $32 \mu\text{m}$ wide at the top and $20 \mu\text{m}$ wide at the base. Fabrication and operation of the glass microfluidic devices has been described previously [9, 12], with the only modification being the expansion of the islet chamber from $300 \mu\text{m}$ to 1 mm to accommodate batches of islets.

Microfluidic reservoirs were purchased from Upchurch Scientific (Oak Harbor, WA). $\sim 100\text{-}\mu\text{L}$ reservoirs (Upchurch N-131) were used for all ports except the perfusion inlet, which used a $1/32''$ tubing interconnect port (Upchurch N-124H). A thin-film resistive heater (Minco Products, Inc., Fridley, MN) was taped to the bottom of the chip to maintain islets, immunoassay reagents, and the reaction channel at 37°C , as monitored by digital thermometer (Fisher). The cell chamber was perfused with BSS using a gas-pressure system to drive flow as described previously [12]. The islet reservoir was open at the top to allow perfusion flow to exit (collected by aspiration). Solution in the islet reservoir was sampled by electroosmotic flow (EOF).

The device utilized a previously described flow gate method of injecting sample onto the separation channel [9, 12, 24]. During the experiment, the islet chamber was held at ground

while the reagent reservoirs were maintained at a floating potential to increase sampling efficiency, as described in the text. Voltage was applied to the waste reservoir via a high voltage power supply (Spellman High Voltage Electronics, Hauppauge, NY). The gate reservoir was connected to ground via a high voltage relay (Kilovac, Santa Barbara, CA). When the relay was opened, sample was allowed to load onto the separation channel. The gate was then returned to ground and separation was performed, after which time another sample was loaded for separation. Laser-induced fluorescence detection occurred 1 cm from the injection point.

Chips were conditioned daily by flowing 1 M NaOH through all channels, followed by deionized water, followed by the experimental solutions for at least 10 min. A chip could be reused after calibration or islet experiments by re-conditioning following the above procedure. During calibration and islet monitoring, 100 μ L of the appropriate solution was placed in all reservoirs (excluding the perfusion inlet) and covered with plastic caps that had access holes drilled into them for insertion of Pt electrodes. Immunoassay reagent concentrations used were either 50 nM FITC-glucagon and 25 nM Ab or 200 nM FITC-glucagon and 100 nM Ab (as discussed in the text) dissolved in reagent buffer. For immunoassays, the applied voltage was 6 kV, with an electric field of 700 V cm⁻¹ in the separation channel. Sample injection time was 0.5 s applied at 5.5 s intervals. Islets were perfused at 1 μ L/min by applying the appropriate He pressure (typically 50–100 psi) to the perfusion system.

Electropherograms were analyzed using previously described software [25]. For islet monitoring, concentrations of glucagon were determined from each electropherogram by comparing FITC-glucagon bound to antibody (B) to free FITC-glucagon (F) peak height ratios to a calibration curve. Calibration curves for off-line experiments (mixing off chip) were obtained by mixing of reagents and glucagon standards in vials and pipetting reaction solutions onto chip reservoirs as discussed in the text. Calibration curves for on-line experiments (mixing in chip) were constructed by pumping different concentrations of standard glucagon in the perfusion media (i.e., balanced salt solution) via the perfusion system into the islet reservoir (Fig. 3b). Calibration were fit to a simple logarithmic function using Prism 3.03 (GraphPad Software, Inc., San Diego, CA).

Isolation and Protocol of Islet Measurements

Islets of Langerhans were isolated from CD-1 mice by a previously described method [26]. To monitor secretion, islets were transferred from culture medium in 4 μ L aliquots and placed in a Petri dish with 3 mL of the balanced salt solution containing 15 mM glucose. Ten islets were then placed in the cell chamber using a pipette while observing using a stereomicroscope. Islets quickly settled to the glass surface and adhered. Care was taken to ensure that islets were placed in a single layer at the base of the chamber. The device was then transferred to an inverted fluorescence-microscope workstation for monitoring the electrophoresis immunoassay, where the islets were perfused with 15 mM glucose for at least 20 min, during which time glucagon was monitored to establish basal levels of secretion. The glucose concentration was then lowered to 1 mM, and secretion continued to be monitored. All error bars are ± 1 standard error of the mean.

Results and Discussion

Off-line Mixing Assay

Initial experiments were directed towards optimizing the CEIA. We were guided in this work by prior studies which had demonstrated a CEIA of glucagon performed in capillaries [27, 28]. Assay development required preparation and purification of FITC-glucagon.

Analysis of the FITC-glucagon reaction mixture by HPLC revealed three main peaks (see Electronic Supplementary Material Fig. S1), two of which corresponded to unreacted glucagon and FITC. The third peak, believed to be FITC-glucagon, was collected and tested for antibody affinity.

Tests were performed by incubating a mixture of presumed FITC-glucagon (50 nM based on UV absorbance measurements), 25 nM Ab, and different concentrations of glucagon standards in glass vials for 10 min and then analyzing the mixture by CE on the chip in Fig. 1. For this analysis, the premixed samples were added to the islet, FITC-glucagon, and Ab reservoirs and no perfusion flow was used. (In this way, premixed samples are injected onto the electrophoresis channel with no dilution.) Representative electropherograms collected with 0 and 100 nM unlabeled glucagon in the incubation mixture are compared in Fig. 2a. Without glucagon present, two peaks are observed corresponding to free FITC-glucagon and FITC-glucagon-Ab complex. Addition of 100 nM unlabeled glucagon completely eliminated the complex peak (Fig. 2a) by competition to yield a single peak that corresponds to free FITC-glucagon. (Identity of this peak was confirmed by comparison to samples with no Ab present.) These experiments confirmed that the HPLC peak was FITC-glucagon, the FITC-glucagon had affinity for the antibody, and electrophoresis conditions were suitable for separation of complex and free FITC-glucagon.

A calibration curve generated by this off-line method shows the classic non-linear curve for competitive immunoassays (Fig. 2b). The limit of detection (LOD) for glucagon was 2 nM. LOD was calculated as the concentration necessary to give a bound to free ratio equal to that of the blank minus 3 standard deviations of the blank. These results demonstrate the viability of the reagents to perform the immunoassay.

These experiments used tricine as the electrophoresis buffer. Initial experiments used HEPES as the electrophoresis buffer because this had been successful for a chip-based CEIA of insulin [9, 12]. Tricine was preferred because HEPES was found to degrade under an applied voltage to yield a fluorescent product that contributed to background. These experiments also used Ab diluted from ascites fluid because this was a low cost source of Ab. Future work may benefit from purification of the Ab used; although we observed no detrimental effects of this preparation.

On-line Mixing Assay

For on-line mixing, 50 nM FITC-glucagon and 25 nM Ab (both dissolved in reagent buffer) were placed in their respective reservoirs while glucagon (dissolved in balanced salt solution) was placed in the islet reservoir (Fig. 1). The islet, FITC-glucagon, and Ab reservoirs were grounded so that EOF could be used to drive these solutions into the mixing channel. Surprisingly, it was found that under these conditions the on-line CEIA for glucagon had a much lower sensitivity and higher LOD than the off-line assay (compare Figs. 2b and 3b, “grounded”). Subsequent study showed that the cause of this low sensitivity was poor sampling from the islet chamber resulting in excessive dilution of sample by reagent, i.e. too much reagent relative to the sample entered the reaction channel (Fig. 1).

Poor sampling efficiency was attributed to low EOF in the sampling channel (see Fig. 1) between the islet chamber and the reaction channel. Although the channel lengths from the islet and reagent reservoirs were equivalent, the high ionic strength of the balanced salt solution that was used to dissolve the sample glucagon (and perfuse islets) caused lower EOF from the islet chamber. EOF in this channel was further reduced by the lower potential drop across the sampling channel because of lower electrical resistance due to high conductivity of the balanced salt solution.

Floating the potential of the reagent reservoirs (FITC-glucagon and Ab) was found to solve this problem. By removing the potential from the reagent channels, the amount of sample entering the reaction channel from the islet reservoir increased by 100-fold. (This increase in sampling was determined by placing fluorophore only in the islet chamber and monitoring the fluorescent signal in the reaction channel). By floating the reagent reservoirs, the majority of flow entering the reaction channel was by electroosmosis from the islet reservoir. Under this condition, reagents enter the reaction channel only by diffusion and possible viscous drag effects.

With significantly enhanced sampling from the islet reservoir, we investigated the utility for on-line immunoassay. Reaction channel residence time was determined to be increased from 80 s to 120 s when floating the reagent channels. When performing the immunoassay with zero-field in the reagent channels, the reagent concentrations (50 nM FITC-glucagon and 25 nM Ab) were diluted below the fluorescence detection limit. This was not surprising as the reagents could only enter the reaction channel by passive effects. Increasing the FITC-glucagon and Ab concentration to 200 nM and 100 nM respectively allowed sufficient reagent to enter the reaction channel to yield good signal in electropherograms (Fig. 3a). As illustrated in Fig. 3b, the sensitivity of the CEIA was comparable to the off-line assay (i.e., B/F ratio changed approximately 3-fold for a concentration change from 0 to 100 nM) as was the reproducibility (RSD < 5% for all concentrations tested). As a result, the LOD was 1 nM, comparable to the offline assay.

Using passive addition of reagents to the reaction channel allowed immunoassays and calibration curves with reasonable sensitivity as long as the reagents were present at sufficiently high concentration. In principle other methods could be used to alleviate the problem of unequal EOF from the sample (i.e., islet) and reagent reservoirs. For example, it would be possible to control the potential on each reservoir separately and therefore tune the flow into the reaction channel. Also, the length of the channels could be adjusted so that the field dropped across each channel gave the desired EOF. The approach of using passive reagent addition is simpler than these alternatives because it relies on a single power supply and fixed channel lengths. Flexibility in reagent addition is retained by control of the concentration in the reservoirs. The method described here has the added advantage of minimizing dilution of sample, which is desirable because of the low amount glucagon secreted from islets.

On-line Islet Monitoring

The device was then applied to monitoring glucose-mediated glucagon secretion from islets. When perfused at 1 $\mu\text{L}/\text{min}$ with 15 mM glucose to provide minimal secretion, groups of 10 islets secreted $1.6 \pm 0.4 \text{ pg min}^{-1} \text{ islet}^{-1}$ of glucagon corresponding to $\sim 5 \text{ nM}$. Stepping down to 1 mM glucose elicited a pulse increase in secretion to $3.8 \pm 0.6 \text{ pg min}^{-1} \text{ islet}^{-1}$ ($n = 4$) followed by a decay to $1.9 \pm 0.4 \text{ pg min}^{-1} \text{ islet}^{-1}$ (Fig. 4). Glucagon secretion measured here is similar to, but somewhat higher than, previous studies [29, 30]. For example, one report shows that glucagon secretion was $0.28 \text{ pg min}^{-1} \text{ islet}^{-1}$ at 15 mM glucose and $0.45 \text{ pg min}^{-1} \text{ islet}^{-1}$ at 1 mM glucose [30]. The higher values observed here could be due to the use of perfusion in this study instead of quiescent solutions in other studies. Paracrine effects play a significant role in glucagon secretion [19, 29, 30]; therefore, the use of perfusion may wash away substances released by other cells in the islet that inhibit glucagon secretion yielding higher values. Higher values may also result from the use of a microfluidic system where losses of hormone are minimized. Finally, since secretion rates are normalized to islet number, use of different islet sizes may account for the differences.

These results show the value of dynamic measurements on a microscale for glucagon monitoring. The pulse of glucagon secretion seen with the change in glucose is similar to a

first phase of insulin secretion and suggests some dynamics of glucagon secretion that have not previously been observed. Therefore, the temporal resolution of measurement offered by the microfluidic system will enable convenient study of the dynamics of glucagon secretion.

It may eventually be possible to monitor glucagon secretion from single islets, as has been done for insulin [9, 12]; however, in attempts to monitor glucagon secretion from single mouse islets in smaller chambers (300 μm diameter) it was found that glucagon was below detectable levels at all glucose concentrations tested (0, 1, and 15 mM). Possibly further miniaturization of the chambers, to minimize dilution, or use of tighter binding antibodies, to improve sensitivity, will enable detection from single islets.

It is interesting to compare the CEIA to a conventional ELISA. Commercial ELISAs have LODs of 0.3 to 10 pM (e.g., assays from BioVendor or RayBiotech). This concentration LOD is far lower than the 1 nM achieved here, making them more suitable for serum measurements, where glucagon concentrations are ~50 pM (but highly variable) [31]. In principle it would be possible to incorporate a preconcentration step to improve the concentration LOD of the CEIA. The microfluidic assay has advantages of reduced sample consumption (less than 1 nL per assay compared to 100 μL for ELISA), speed (5 s compared to 6–24 h for ELISA), and reagent cost (a few cents per assay compared to \$4 to \$12 for ELISA). These advantages make the microfluidic CEIA well-suited for the in vitro research applications which require many assays at small volumes. The experiments illustrated by Figure 4 involved 720 immunoassays which would be cost prohibitive with commercial ELISAs.

Conclusions

A microfluidic system was demonstrated for on-line chemical monitoring of glucagon secretion from living islets of Langerhans. The automated device allowed for real-time evaluation of islet responses with 6 s temporal resolution based on perfusion, reagent addition, and automated CEIA. Initial results reveal dynamics of glucagon secretion not easily studied by other means. The system alleviates many of the disadvantages of traditional immunoassay methods, specifically off-line analysis and manual sample handling. Use of this method should facilitate investigations of the role of glucagon in obesity and diabetes. Further it helps validate and expand the concept of microfluidic CEIA as a tool for monitoring cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Microfluidic device to continuously monitor glucagon release from islets of Langerhans. Channels (lines) and access holes (circles) are drawn to scale. All channels were 6 μm deep. Electrical connections are shown as dashed lines. Perfusion inlet was connected to external gas-pumped liquid system.

Fig. 2.

Electropherogram and calibration curve obtained from offline mixing of reagents. (a) Equal volumes of a solution containing 50 nM FITC-glucagon, 25 nM Ab, and 0 (solid) or 100 nM glucagon (dashed) were placed in all three sample reservoirs and injected onto the separation channel for 0.5 s with a separation voltage of -6 kV. (b) Calibration curve for 0–100 nM glucagon mixed with 50 nM FITC-glucagon and 25 nM Ab. Points are the average from 20 consecutive electropherograms, and error bars are ± 1 standard deviation.

Fig. 3. On-line reagent mixing electropherogram and calibration curves obtained from microfluidic device. (a) Electropherogram collected during on-line monitoring of islet secretion. FITC-glucagon complexed with Ab (B) and free FITC-glucagon (F) are denoted. (b) Comparison of calibration curves from 0 to 100 nM glucagon obtained by grounded (circles) and floating potential (squares) FITC-glucagon and Ab reservoirs. Grounded system used 50 nM FITC-glucagon and 25 nM Ab; zero-field used 200 nM FITC-glucagon and 100 nM Ab. Points are the average from 20 consecutive electropherograms, and error bars are 1 standard deviation.

Fig. 4. Glucagon release measured from islets of Langerhans on a microfluidic chip. (a) Plot of average glucagon secretion. Glucagon concentration was determined from B/F of individual electropherograms and online calibration; average basal levels were determined by measurement of secretion 5 min prior to stimulation. Step change in glucose is denoted by arrow. Error bars ($\pm$ SEM) are shown every 5th data point for clarity. $n = 4$. (b) Comparison of glucagon secretion at 15 mM (basal) and 1 mM glucose (as determined by maximum value). * indicates difference from control with $p < 0.01$.