Amperometric glucose sensor for real time extracellular glucose monitoring in microfluidic device

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ABSTRACT

We have combined a microfluidic network and developed a miniature glucose sensor to measure glucose consumption of single cardiomyocytes in a confined extracellular space. The thin film glucose sensor was integrated into a 360 picoliter extracellular space. We used Pt thin film electrodes (20x100 μm^2) as a counter electrode, and a needle type reference electrode (0.45 mm diam) placed in the cell loading port of the microfludic device. Single cardiac cells were trapped in the sensing volume using pressure gradients. Using miniature mechanical valves allowed us eliminate the interference of flow artifacts on the detection of glucose. We have demonstrated the feasibility to measure the glucose consumption of single cardiac myocytes in an enclosed confined extracellular volume using of miniature glucose sensitive electrodes fabricated by spin-coated deposition.

Keywords: glucose sensor, glucose oxidase, spin-coated deposition, microfluidic device, cardiomyocytes.

INTRODUCTION

Microfluidic devices are becoming wide spread in different areas of biology and medicine [1-3]. Microfluidic based lab on a chip devices provide several advantages for cellular analysis systems and cells can be confined in a chemically controlled in-vivo like microenvironment comparable to the cell size. Although, progress has been made incorporating sensors into lab-on-a-chip devices there is a need to develop microsensor arrays for multiple analyte sensing. Glucose and oxygen consumption, acidification and lactate production rates are the most important variables which characterize the metabolic activity of living cells. Measuring multiple metabolites will allow us to monitor the metabolic response of cells chemicals, drugs or toxins in lab on a chip device. The microsensor and microfluidic confinement is necessary to measure of metabolic activity in vicinity of single cells in sub nanoliter volumes reliably with a sufficient signal to noise ratio. To measure glucose consumption rates in small cell culture volumes, we have developed a microfluidic device with active valves, which confines single cardiac myocytes in sub nanoliter volumes on a microfabricated planar thin film glucose sensing electrode. The glucose sensing electrodes were fabricated using thin film platinum microelectrodes spin-coated with glucose oxidase (GOx). Such sensors possess a high sensitivity, long-term stability and can operate in different cell growth media. The development and miniaturization of a stable glucose biosensor are not only of great interest to monitor cell physiology but may lead to an alternative approaches to measure blood glucose levels.

RESULTS AND DISCUSSIONS

A glucose biosensor based on the electrochemical detection of enzymatically generated H_2O_2 was constructed by the spin-coating glucose oxidase and glutaraldehyde cross-linking onto Pt electrodes, followed by an additional coating with a Nafion protection layer to obtain long term stability and sensitivity (Fig.1).

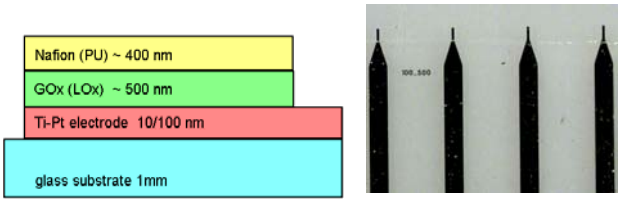

Fig.1. (a) Cross-sectional schematic of the thin-film glucose sensor. (b) Optical image of four glucose sensors on a glass substrate. The working area of sensors was 50x500 and 100x500 μm^2.

Fig.2 shows the layout of the microfluidic device overlaid onto the sensing electrode configuration. The base electrodes for the glucose sensor were fabricated by vacuum evaporation of a Ti adhesion layer and a Pt layer on glass substrates (24x24mm) and standard photolithography. The working areas of electrodes were 50x500 and 100x500 μm^2. The electrode array consists of seven independent electrodes. Usually three of them were used as glucose sensors and were positioned in the cell sensing volume. The others (bare Pt films) electrodes were used as counter electrodes. A needle type electrode (DriRef-450 from WPI Inc., diameter 0.45 mm) was inserted in the cell loading port and used as reference electrode.

The enzyme solution for glucose sensitive electrodes was prepared based on the procedure described by Eklund et al (4). In our protocol, the glucose oxidase

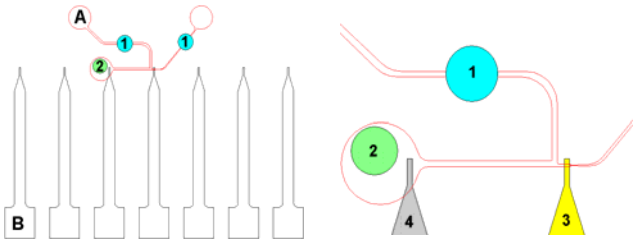

Fig.2. Schematic overview of a NanoPhysiometer for glucose consumption measurements from single CM cells in sub-nanoliter volumes: (A) micro-fluidic channels array; (B) microelectrode array; (1) mechanical valve; (2) Ag/AgCl reference electrode; (3) glucose sensor; (4) Pt counter electrode. The inset (a) shows a detailed view of microelectrodes in the detection volume

(GOx) film solution was prepared by dissolving 2.5 mg GOx and 25 mg of BSA in 250µL of 1mM PBS containing 0.02% v/v Triton X-100. After the BSA and GOx were completely dissolved, 15 µL of 2.5% glutaraldehyde solution was added and thoroughly mixed with a Vortex shaker for 10 min. Each time the fresh enzyme solution was prepared for the deposition of glucose oxidase coated electrodes.

Different types of polymer protective membranes were tested to stabilize the characteristics of the glucose sensors. The requirement for the polymer membranes is the permeability for glucose and oxygen. In our investigation we tested polyurethane (PU) and Nafion membranes because of their good glucose diffusion-limiting behavior and biocompatibility [5]. 3-4 pellets of PU (~ 0.5 g) were dissolve in 5 ml of tetrahydrofuran and stirred for over 2 hours at room temperature. The Nafion films were obtained from 5% Nafion solution.

The electrodes were functionalize by spin coating the enzyme and the protective membrane on freshly cleaned platinum electrodes. The substrates were cleaned by sonicating in ethanol and in acetone and rinsed with running DI water. The enzyme solution (100 µL) was added onto the substrate with a pipette. The substrate was then spun at 1500 rpm for 30s with a conventional spin coater (Laurell Model WS-400A-6TFM/LITE). The resulting film thickness was typically ~0.4 µm. After spin coating the GOx films were dried at room temperature during 1 hour.

The films of polymer membranes were also deposited by spin coating. For the Nafion films the actual conditions were: dispense a 100 µl drop of solution onto the electrodes, spin at a speed = 1000 rpm, acceleration = 300 rpm s^{-1} and time = 30 s. The resulting Nafion film is transparent with a typical film thickness of ~ 0.4 µm. The PU films were deposited at 3000rpm during 30s, which resulted in a 0.7 µm thick film.

For characterizing the glucose sensors in a beaker experiment the surface of the electrodes except working area (100x500 µm, fig.1b) were covered with a thin PDMS film (100 µm) After fabrication the glucose sensors were stored at 4°C before use.

The microfluidic network was fabricated from PDMS (polydimethylsiloxane) by replica molding, using photoresist on a silicon wafer as a master. The master was fabricated by spinning a 20 µm thick layer of photoresist (SU-8 2025, MicroChem Corp, Newton, MA) on a 3"diameter silicon wafer (Nova Electronic Materials, Ltd, Carrolton, TX) and by exposing it to UV light (160 mJ/cm^2) through a metal mask using a contact mask aligner. The photoresist was processed according to the manufacturer's recommendation on the datasheet. An optional 30-minute hard bake at 200°C was performed on a hot plate to increase the durability of the resist. PDMS prepolymer was mixed with the curing agent in a 10:1 ratio by weight and degassed in a vacuum chamber for 20 min. The master was placed in a clean Petri dish, which was filled to a height of approximately 1 cm with PDMS and cured in an oven for 4 hours at 70°C. After curing, the elastomer was mechanically separated from the master and cut into discrete devices. Access holes for the fluidic connections were punched into the PDMS using sharpened blunt-tip 18 gauge needles.

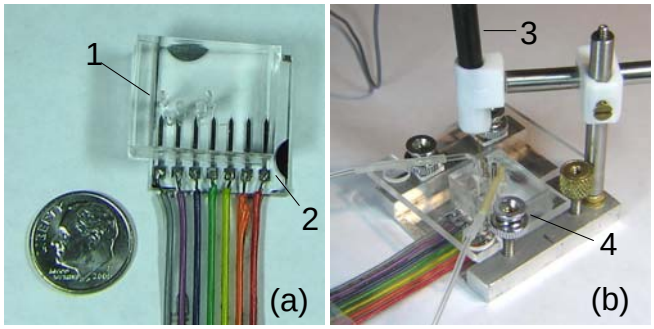

Fig. 3. (a) Alignment of glass substrate with glucose sensors (2) and PDMS microfluidic (1) device (the channel height – 40mm, the cell trap volume – 0.36 nL). (b) Photo of the clamp mechanism (4), which ensures reliable seal between PDMS microfluidic device and glass substrate with thin film glucose sensors; (3) - Ag/AgCl reference electrode diam. 0.45mm.

The PDMS microfluidic device was manually aligned relative to the glucose sensitive electrodes (fig.3a) with a stereo microscope. The PDMS device was sealed to the glass substrate by auto-adhesion (fig.3a), and stabilized with a mechanical clamp (fig. 3b). Glass capillaries were inserted into the access holes to connect the microfluidic channels to syringes/pumps using standard microtubing (0.5mm inner diameter, Cole Parmer). For the trapping of cells in the sensing volume we controlled the syringes connected to the output and control port by hand. A microsyringe pump ''Micro 4'' (WPI, Sarasota, FL) was used to control the flow of solution during the calibration of the glucose electrodes in the microfluidic devices.

In order to precisely control residual flows after stopping the perfusion in our micro fluidic devices, we developed miniature mechanical screw valves [7]. Our design allowed us to place easily two valves in close

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proximity to the cell trap (fig.2). The valves were fabricated by drilling a pocket hole above the microfluidic channel into the PDMS. Into the pocket hole we inserted an oversized threaded sleeve and a screw which allowed us to pinch off the microfludic channel beneath the screw.

There are two major reactions involved for electrochemically sensing glucose. The glucose oxidase (GOx) oxidizes the glucose and produces gluconic acid and hydrogen peroxide as described by the following reaction [8-9].

$$\text{Glucose} + O_2 + \text{GOx} \rightarrow \text{gluconic acid} + H_2O_2$$

H_2O_2 is then oxidized electrochemically on the Pt electrode resulting in a current proportional to the glucose concentration.

$$H_2O_2 \rightarrow (0.6\text{V Pt vs Ag/AgCl}) \rightarrow 2H^+ + O_2 + 2e^-$$

The most important advantage of the amperometric detection of H_2O_2 in contrast to a Clark type oxygen electrode detecting the oxygen production is the simplicity of the electrode fabrication, reduced cross-talk and the possibility of miniaturization.

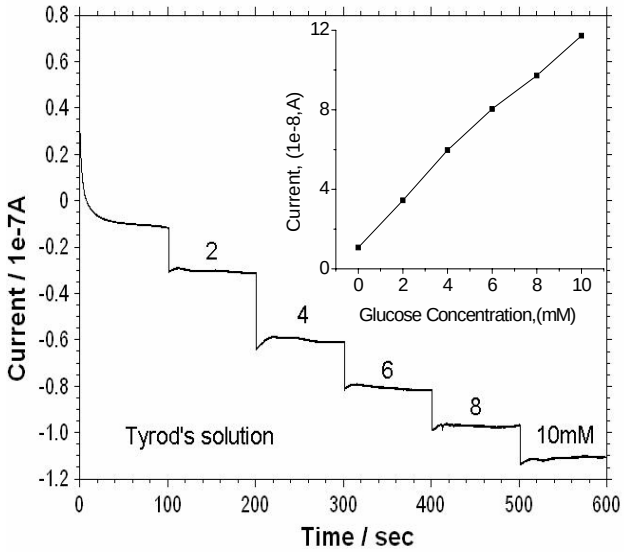

Fig.4. Current-time plots as a function of glucose concentration in Tyrod's (pH7.4) solution. Inset: calibration curve of sensors as a function of glucose concentration

Fig. 4 shows the response of the amperometric glucose sensor obtained by adding glucose to stirred Tyrod's solution. The potential of the working electrode was maintained at 0.6 V with respect to Ag/AgCl reference electrode. The current shows a stable constant stepwise increase in current. The resulting calibration curve for glucose over the physiological concentration range form 0–10mM is presented in the inset of Fig.4. The temporal response of the glucose electrode to a change in glucose concentration is reasonably fast (within 10 s).

The operational stability of our glucose sensors were tested by continuously monitoring the steady-state response in Tyrod's solution at glucose concentrations of 6 and 8 mM at room temperature (Fig.5). With the exclusion of slight low frequency fluctuation which we attribute to temperature fluctuations, the current response didn't change appreciable during a 1 hour period of continuous use, which is sufficient to conduct several experiments with cardiac myocytes. The long-term stability was investigated by successive calibrations. No appreciable loss in glucose sensitivity was observed up to about 3 month.

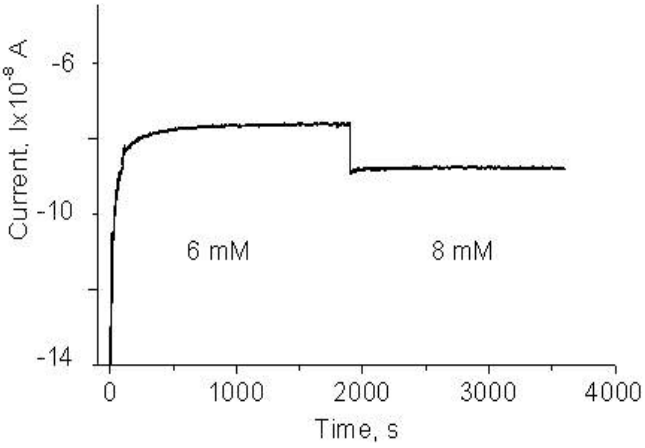

Fig.5. Investigation of long term stability of the glucose sensor in Tyrod's solution (pH7.4) with 6 and 8 mM glucose concentration.

The microfabricated glucose sensors were used in conjunction with a microfluidic device (NanoPhysiometer (NP)) that allows trapping the single cardiac myocytes in a confined sub-nanoliter volume adhered to the microfabricated glucose sensitive thin film electrodes on a glass substrate. The glass slide provides the base layer for the microfluidic network made of polydimethylsiloxane (PDMS) with integrated valves to allow control of the fluidic access to the cell detection volume (Fig.6and 3b).

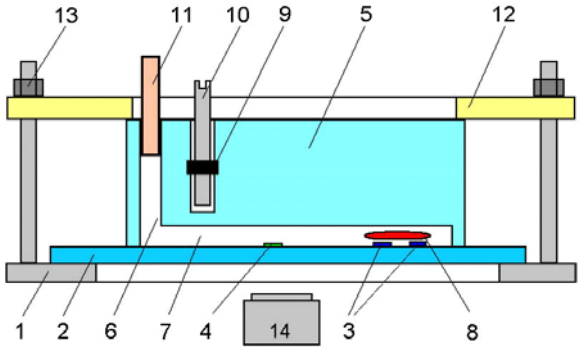

Fig.6. Cross-sectional schematic of the completely assembled NanoPhysiometer. A clamp ensures mechanical stability of the seal between the PDMS microfluidic device and the substrate with the electrode arrays. 1-aluminum

base; 2-glass substrate; 3-glucose sensitive electrode; 4-Pt counter electrode; 5- PDMS microfluidic device; 6-input port; 7-microchannel; 8-single cardiac myocyte cell; 9-thredded sleeve; 10-microvalve screw; 11-Ag/AgCl reference electrode; 12-acylic cover; 13-screw to adjust pressure; 14-objective

The glucose sensitive electrodes were tested in a microfluidic environment to compare their characteristics with the beaker experiments. Fig.7 show that sensitivity and stability of spin coated glucose sensor in the microfluidic channel (100 × 40 μm^2) were practically the same as those obtained from macroscopic experiment in beakers.

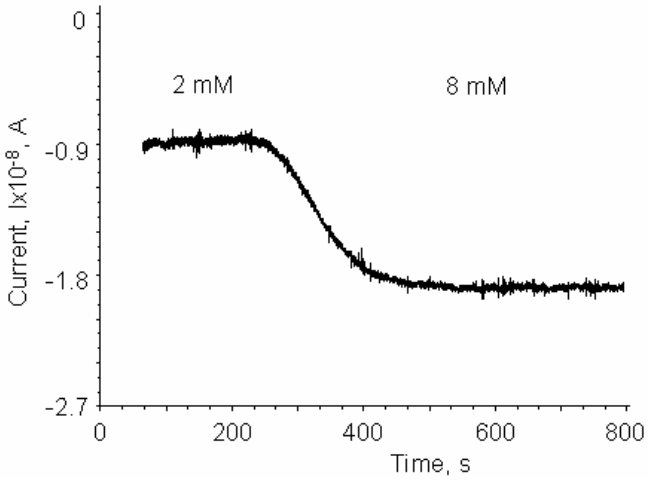

Fig.7 Calibration of glucose sensor in microfluidic channel for two Tyrod's solution with different concentration of glucose (2 and 8 mM). The rate of flow is 2 nL/s.

The design of our microfluidic network allows us to trap single myocytes and to quantify glucose concentration changes in 360 pL volume. Fig.8 shows an example of the measurements of glucose consumption from cardiac myocyte cells in the subnanoliter volume in Tyrode's solution (10 mM glucose, 0.5 mM Ca^{2+}). After initiating the amperometric measurements and closure of the microvalves we observed a rapid change in the current for a period of ~100 s. After this stabilization period we observed a linear decrease of the anodic current over time which corresponded to reduction of the glucose concentration in the sensing volume of the Nanophysiometer. After the removal of the cell, as indicated by the step in the current time plot (Fig 8), the anodic current (glucose concentration) was constant demonstrating that the consumption of glucose by the electrode is negligible compare to the consumption by the myocyte.

In conclusion, we have developed a microfluidic device platform to measure the glucose consumption of single cardiac myo-cytes in confined sub-nanoliter volumes. We plan to use the Nanophysiometer to investigate the metabolism in isolated cardiac cells under condition comparable to ischemia.

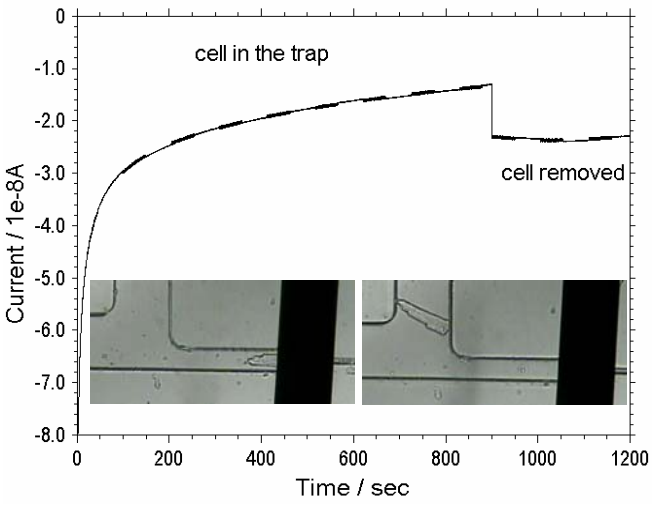

Fig.8. Measurement of the glucose consumption from single cardiac myocytes in the Nanophysiometer. The dark region in the image is the spin coated glucose sensitive electrode (GOD ~ 0.4 μm, Nafion ~ 0.4 μm) with working dimensions of 40x100 μm^2. The inset shows a detailed view of the myocyte in and the removal of the measurement volume during the experiments.

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