On-Chip Ion-selective Microesensor for Evaluation of Mitochondrial Membrane Potential

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ABSTRACT

The mitochondria have proven to be at the fulcrum point generating energy in living systems and regulating the aging process and diseases such as cancer, diabetes, and Parkinson's Disease[1,2]. Therefore, there is a need for tools to study its regulation in a controlled environment for potential clinical and scientific applications. For this aim, an on-chip ion-selective microsensor was constructed in a microfluidic environment to monitor functional status of cells by assessing mitochondrial membrane potential. The concentration of isolated mitochondria (Heb7A) used in the measurement was 0.3 ng/mL, four orders of magnitude smaller conventional assays (3 mg/mL). We demonstrated that changes in the mitochondrial membrane potential are clearly measured in response to a barrage of substrates and inhibitors of the electron transport chain.

Keywords: mitochondria, microsensor, microfluidics, ion-selective electrode

1 INTODUCTION

Mitochondria is often referred to as the powerplant of our body, becuase these organelle synthesize cellular ATP by converting ADP to generate energy. In addition to being the main energy producers, mitochondria play a pivotal role in regulation of cellular functions, apoptosis, and homeostatis and carcinogenesis. In order to improve our understanding of the biochemical nature of these relationships, there is a need for improved instrumentation to study and diagnosis mitochondrial properties and function. The mitochondrial membrane potential ($\Delta\Psi_m$) across the inner mitochondrial membrane is the most important property providing a broad overview of the metabolic health and funtional status of cells. Numerous methods have been used to estimate $\Delta\Psi$m including flourescenct methods and electrochemical probes. Since the physical impalement of the mitochodrial membrane with needle-type electrodes is challenging, lipophilic cat ions are widely used as indicator probes, whose distribution is related to $\Delta\Psi_m$ through the Nerst equation. Kamo et al. reported an ISE membrane potential electrode using tetraphenylphosphonium (TPP^+) ions, a lipid-soluble cation, and found that TPP^+ can permeate through mitochondrial membranes with 15 times faster diffusion coefficient than other cations such as DDA^+ (debenzyldimethyl ammonium)[3]. Since the accumulation of TPP^+ ions into the mitochondrial matrix is related to $\Delta\Psi_m$ through the Nernst equation and volumetric factors, its value can be determined from the concentration of TPP^+ ions.

In this paper, we present the first on-chip tetraphenylphosphonium (TPP^+) ISE microsensor and demonstrate its application in the measurement of $\Delta\Psi_m$ in a controlled micofluidic environment. Details of the fabrication of the microfluidic TPP^+ selective sensor using multilayers of polydimethylsiloxane (PDMS) and preparation of a TPP^+ selective membrane and a liquid juntion reference electrode are described (Fig. 1). A continuous measurement of $\Delta\Psi_m$ with isolated mitochondria (Heb7A) is performed and demonstrated with the fabricated devices.

2 METHODS

2.1 Fabrication

The microfluidic TPP^+ selective sensor was constructed on a microscope cover glass with PDMS layers. The fabrication steps are schematically illustrated in Fig. 1. Cover glasses were cleaned in Piranha solution (mixture of sulfuric acid and hydroperoxide with the ratio of 7 to 3) overnight followed by organic solvent cleaning. First, a thin film of Ti (30 nm) was deposited

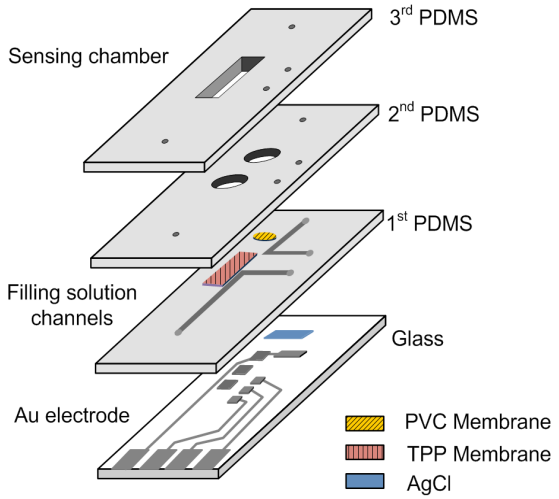

Figure. 1: Illustration of layer-by-layer sensor construction with three PDMS layers on the glass substrate.

as a seed layer and then Pd (50 nm) and Ag (2.5 mm) were deposited on the cleaned glass substrate using an electron-beam evaporator (Airco/ Temescal CV-8). Photolithography was performed with positive photoresist (Shipley 1827) for patterning silver electrodes on the glass substrate. Shipley 1827 was spread out by using a spin coater at 3500 rpm for 30 sec. with 3 µm thickness and soft-baked at 90 °C for 10 min. The spin-coated glass substrate was exposed to UV-light for 30 sec. at 6 mW/cm^2. After a post-bake at 120 °C for 3 min., the exposed photoresist was removed in developer (MF-319, Microposit). Chlorination of silver (for Ag/AgCl electrode formation) was carried out electrochemically or chemically for both the reference and working electrodes. The former was performed in 0.1 M HCl solution at a constant current of 5 mA/cm^2 for 4 min., and for the latter the silver coated glass substrate was dipped into 0.1 M $FeCl_3$ solution for 40 sec. without current flow. It was found that the electrode chlorinated with the chemical method in 0.1 M $FeCl_3$ solution works best in terms of robustness and surface morphology. For the sensor characterization and test, only the sensor chlorinated by the chemical method was employed through the study.

The PDMS layer was prepared by a mixture of PDMS prepolymer (Sylgard 184, Microchem) and a curing agent at a ratio of 10:1. A 7 g mixture was poured onto a 4" silicon wafer used as a substrate. After curing PDMS in a hot oven at 90 °C for 15 min., the cured PDMS with thickness of 1 mm was cut into the size of a cover glass and stripped out from the wafer. With a surgical blade and a flat-end needle, two L shaped inner filing solution reservoirs were cut in accordance with electrode sensing areas on the glass substrate. For the liquid junction Ag/AgCl reference electrode, polyvinyl chloride (PVC) protective membrane was constructed to keep the concentration of chloride ions in the 3M KCl inner filling solution constant. The PVC membrane solution prepared with 50 wt% PVC powder and 50 wt% 2-nitrophenyl-octylether was disolved in tetrahydrofuran (THF, Fa. Fluka) and the mixture was dropped on to a cleaned glass substrate followed by curing at room temperature. The cured membrane was transferred onto the top of the L-shaped reservoir and glued with THF. The TPP^+ selective membrane was prepared with a mixture of 4.4 ml of THF, 0.36 ml of dioctyl phthalate, 0.15 g of PVC and 6 mg of Na^+ TPB^- (teteraphenylboron), and poured onto a glass substrate. The mixture was evaporated slowly at room temperature for a few hours. The membrane was carefully glued to the top of the TPP^+ reservoir with THF. The 2nd PDMS layer having two openings and four access holes was placed on top of the membranes to secure the bonding of membranes between PDMS layers by pressing down the membranes. Two inner filling solutions (10 mM TPP^+Cl^-, 3M KCl) were introduced into two L-shaped reservoirs through access holes by means of syringes and rubber tubes. Care should be taken during the introduction of the inner filling solution to avoid air bubbles, which lead to an open sensing circuit. The membranes in the two opening areas are exposed to the medium. For the sensing chamber, the 3rd PDMS layer (4 mm thick) was bonded to the top of the 2nd layer with a chamber volume of 85 µL.

3 RESULTS

3.1 Calibration

The sensor calibration was performed at various concentrations of TPP^+Cl^- solution ranging from 10 µM to 10 mM in both respiratory buffer and 0.1 M NaCl solutions at 25 °C while monitoring potential differences between working electrode and reference electrodes. Three working electrodes were integrated in the same chip for the future use of parallel measurements with oxygen respiration, demonstrating the potential for the integration of different kind of sensors in the same chip. In the test, one of the three electrodes was used for the measurement. After filling the test chamber with 71 µL of 0.1 M NaCl (or respiration buffer), 2 µl of various concentrations of TPP^+Cl^- solution was added into the test chamber successively using micropipettes. The measured potential from the fabricated sensors is plotted vs. time in Fig. 2(a) and the arrows indicate the addition of 2 µl TPP^+ solution. Upon the addition of solutions, signal spikes were recorded due to the physical impact of dropping solutions into the chamber medium. Once the potential signal stabilized, it maintained a constant level showing that no significant evaporation effect occured during the test. The signal stablized within 25 sec., which is considered to be the response time (t_{90}) of the electrode.

After each measurement, the sensing chamber

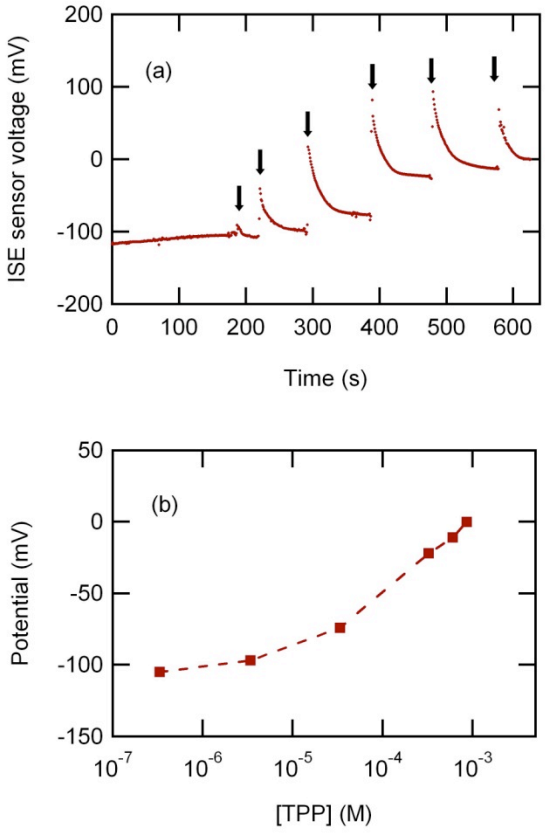

Figure 2: Calibration curve of TPP sensor. (a) Characterization was performed in respiration buffer solution (25°C, pH 7.2) using fabricated TPP+ microelectrode sensors with successive additions (arrows) of 2 µL TPP+ solution (10µM to 10 mM) resulting in final concentration of 0.3 µM, 3 µM, 30 µM, 300 µM, 600 µM, 900 µM respectively. (b) Sensor response in log scale.

including TPP^+ selective membrane was cleaned with DI water several times. When inhibitors and substrates are used during the mitochondrial measurement, 50% ethanol was used to rinse the chamber to avoid interferences from residues on the membranes.

3.2 Measurement of mitochondrial membrane potential

The evaluation of the mitochondrial membrane potential was performed with human mitochondria (Heb7A) in respiration buffer (225 mM Mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, 5 mM KH_2PO_4, pH 7.2). The measurements were repeated 4 times with freshly prepared mitochondria to confirm the performance and the reproducibility of the sensor. The results of the measurements showed reproducible responses under similar conditions. We used 25 ng of isolated mitochondria in 85 mL for the test resulting in a final concentration of 0.29 ng/mL. The mitochondrial membrane potential ($\Delta\Psi_m$) can be determined using[4]:

$$\Delta\Psi_m = \frac{RT}{F}\ln\frac{V_0[TPP^+]_0/[TPP^-]_t - V_t - K_0P}{V_mP + K_iP}, \quad (1)$$

where $[TPP^+]_o$, $[TPP^+]_t$ represent TPP^+ concentration in the test chamber before the addition of mitochondria and at time t respectively. V_o is the initial buffer volume in the chamber and V_t represents the final volume in the chamber which includes the total mass (in mg) of mitochondrial protein (P) added in the assay. For our purposes, the mitochondrial matrix volume (V_m) was assumed to be equal to 1 µL/mg protein. The partition coefficients describe the innate binding and accumulation of the cationic TPP^+ ion to the matrix (K_i) and external (K_o) faces of the inner membrane and are given values of 7.9 µL/mg and 14.3 µL/mg, respectively[5].

The respiration chamber was filled with an initial volume of 71 µL respiration buffer. Once the plot baselined to zero, we introduced 5 mL of 100 µM TPP^+Cl^- solution to provide a working concentration. We purposely kept the TPP^+ concentration $[TPP^+]$ below 10 µM to prevent inhibition of respiration[6]. After stabilization, 5 µL of freshly isolated mitochondria (5 ng/µL) was added to the chamber. This was quickly followed by a dramatic decrease in the external concentration of TPP^+, indicating an uptake of the TPP^+ ion into the mitochondrial matrix through the inner membrane. This absorption was stimulated by the addition of 10mM pyruvate (P) and 5 mM malate (M). These Complex I substrates induce the pumping of H^+ ions out of the matrix (synchronously with the shuttling of electrons through the ETC) into which TPP^+ can rush

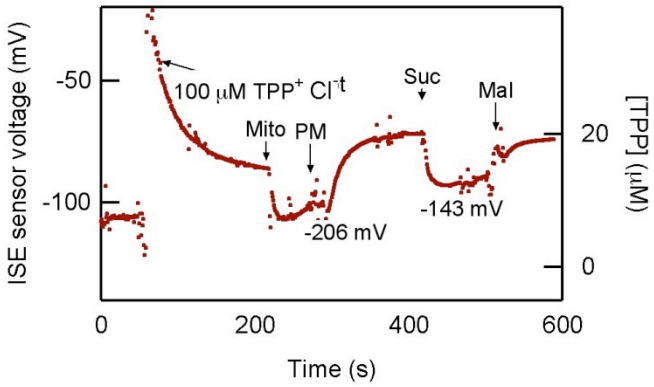

Figure 3: Measurement of $[TPP^+]$ and inferred $\Delta\Psi_m$ with isolated mitochondria. Arrows indicate successive addition of 100 µM TPP^+Cl^-, 5 µL of 5 ng/µL isolated mitochondria (mito), 10 mM pyruvate (P), 5 mM malate (M), 4 µM rotenone (Rot), 10 mM succinate (Suc), 5 µM malonate (Mal). See text for discussion. (Note the [TPP] scale is not linear.)

in to fill the loss of positive charge. This is quickly reversed upon addition of 4 μM rotenone (Rot) to the chamber. Presumably, the positive H^+ charge builds up in the matrix once Complex I is inhibited which leads to the expulsion of TPP^+ ions back into the sensor chamber; as illustrated in the dramatically increased [TPP^+] seen in the plot. Complex II was then serially stimulated with 10 mM succinate (Suc) to much the same effect- the external [TPP^+] was decreased as it was pulled into the mitochondrial matrix. Accordingly, the addition of 5 mM malonate (Mal), the Complex II inhibitor, caused the release of TPP^+. The reproducibility and sensitivity of our device provides evidence for the viability and validity of our protocol to measure the mitochondrial membrane potential in tiny sample amounts.

4 CONCLUSIONS

In conclusion, we successfully constructed the microfluidic mitochondrial membrane potential sensor by using microfabrication techniques. The fabricated TPP^+ selective electrode showed excellent Nerstian response to changes in the concentration of TPP^+ ions. The continuous measurement of mitochondrial membrane potential using isolated mitochondria exhibited that this sensor can be a useful analytic tool for studies on mitochondrial energetic and further cellular respiration as well. Our assay requires four orders of magnitude less concentraion and two orders of magnitude less volume than standard methods, indicating the potential of microtechnology and nanotechnology for ultra-sensitive diagnostic and scientific studies of mitochondrial membrane potential, eventually even down to the level of assying a single mitochondria.

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