BIOASSAY CHIP FOR EVALUATION OF MITOCHONDRIAL MEMBRANE POTENTIAL WITH INTEGRATED ION-SELECTIVE MICROSENSORS

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ABSTRACT

In this paper, we present the first on-chip tetraphenylphosphonium (TPP^+) ion selective electrode (ISE) microsensor and demonstrate the measurement of the mitochondrial membrane potential ($\Delta\Psi_m$) in a microfluidic environment. A continuous measurement of $\Delta\Psi_m$ with isolated mitochondria (Heb7A) is performed with the fabricated devices. The concentration of isolated mitochondria used in the measurement was 0.3 ng/mL, four orders of magnitude smaller than the concentration used in conventional assays (3 mg/mL). The changes in $\Delta\Psi_m$ and TPP ion concentration are measured in response to successive additions of substrates and inhibitors of the electron transport chain.

KEYWORDS: Mitochondria, Ion selective electrode, Lab-on-a-chip, Microfluidics

INTRODUCTION

The ion transport through transmembrane proteins (ion-channels) in the cell membrane generates electrochemical gradient across the membranes developing cellular membrane potential. This electrochemical potential differences across membranes regulate cellular metabolism and functions indicating the healthy status of cells. Here, we study mitochondrial membrane potential, because mitochondria are major energy sources of cells by converting ADP into ATP. In addition to being the main energy producers, mitochondria play a pivotal role in regulation of cellular functions, apoptosis, and homeostasis and carcinogenesis[1,2]. ATP synthesis takes place in the mitochondrial inner membrane through ion transport chain by using electrochemical gradient of protons. The proton pumps in the inner membrane which called Mitochondrial Complex I –IV control the in/outflux of proton modulating the membrane potential. Therefore monitoring changes in the mitochondrial membrane potential provides a great deal of information of mitochondrial properties and better understandings of biochemical relationships. In addition, the use of on-chip ISE devices has the potential for low cost, label-free, real-time, and facile integration with lab-on-a-chip type systems, which motivates our work in this paper.

THEORY

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.

EXPERIMENTAL

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 ℃ 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(left) 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 including TPP^+ selective membrane was cleaned with DI water se-

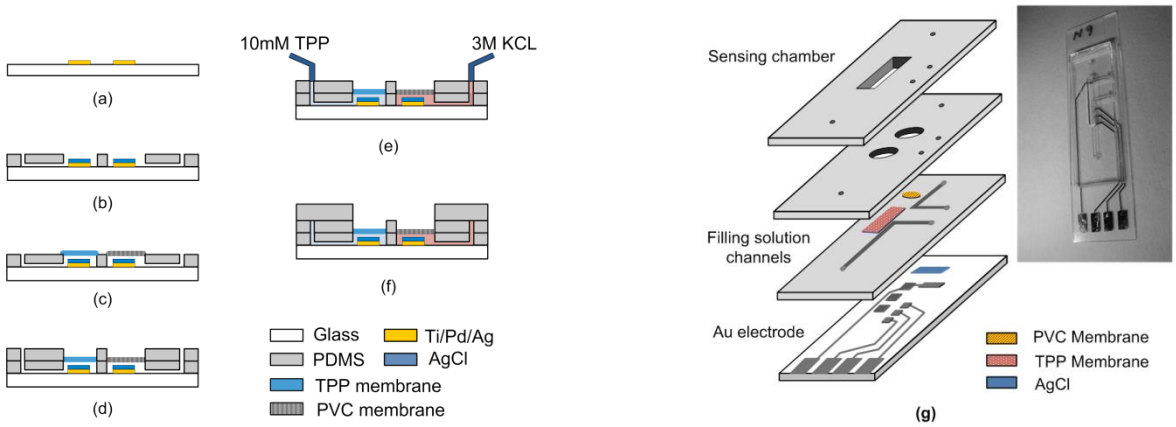

Figure 1: Schematics of Microfabrication and sensor design . (a) Metallization with Ti/Pd/Ag (b) chlorination of Ag to deposit Ag/AgCl (c) ISE selective membrane and AgCl protective membrane deposition (d) 2nd PDMS layer bonding (e) filling solution introduction (f) final assembly with 3rd PDMS layer (g) layer by layer sensor design (inset) a photo of the fabricated microsensor

veral 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.

RESULTS AND DISCUSSION

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

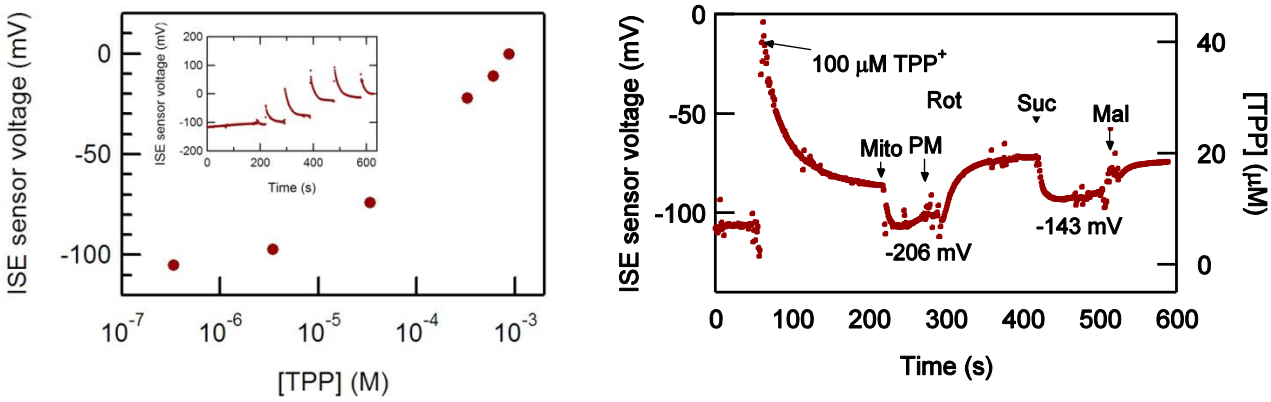

Figure 2: (left) Calibration curve of TPP performed in respiration buffer solution (25°C, pH 7.2) with successive additions (inset) of 2 µL TPP+ solution form 10µM to 10 mM . (right) Continuous measurement of [TPP+] and inferred ΔΨm with isolated mitochondria. Arrows indicate successive addition of 100 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.)

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 results of a typical assay are shown in Fig. 2 (right). The respiration chamber was filled with an initial volume of 71 μL respiration buffer. Once the plot baselined to zero, we introduced 100 μM TPP+Cl- solution to provide a working concentration. We purposely kept the working concentration of [TPP+] ~10 μM to prevent inhibition of respiration[6]. After stabilization, isolated mitochondria (5 ng/μL) was added to the chamber. The fresh mitochondria quickly took in TPP^+ from the chamber due to its value of $\Delta\Psi_m$, resulting in a lower TPP^+ concentration in the chamber as measured by the ISE. However, as the mitochondria consumed substrates in the respiration buffer, the substrate concentration became depleted, and the magnitude of $\Delta\Psi_m$ began to decrease slowly as a result, causing a slow increase in [TPP^+] in the chamber. This slow decrease in the magnitude of $\Delta\Psi_m$ was temporarily halted by the additional of complex I substrates PM, which allowed the mitocondria to increase the magnitude of $\Delta\Psi_m$ through consumption of these substrates. While there are transients in the data, the slow decrease in the magnitube of $\Delta\Psi_m$ is clearly halted by the addition of PM. The complex I inhibitor Rot halts the mitochonrdial consumption of PM, leading again to a slow decline in the magnitude of $\Delta\Psi_m$ (hence an increase in [TPP].) The addition of Complex II substrate Suc allows the mitochondria again to increase the magnitude of $\Delta\Psi_m$ (hence decreasing [TPP].) The addition of complex II inhibitor Mal stops the consumption of Suc, causing again a slow decrease in the magnitude of $\Delta\Psi_m$ (thus increasing [TPP].)

These measurements clearly demonstrate the ability of the on-chip ISE to assay meaningful mitochondrial responses to various biochemical stimulants in a controlled, microfluidic environment. We turn next to the device reliability, reproducibility, and sensitivity.

CONCLUSION

We successfully constructed a 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 with a linear relation in logarithmic scale with respect to the membrane potential. 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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