IMPEDANCE BASED BIOSENSOR WITH DIELECTROPHORESIS CONCENTRATION FOR CARDIAC MYOCYTE HYPERTROPHY SENSING

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ABSTRACT

Cardiac hypertrophy is an established and independent risk factor for the development of heart failure and sudden cardiac death that may be regulated by growth factor such as Endothelin-1 (ET-1). The majority of existing techniques to monitor hypertrophy *in vitro* is based on florescence probes designed to show morphological and biochemical alterations indicative of cardiomyocyte hypertrophy [1]. In this paper, a new cardiomyocyte-based impedance sensing system with the assistance of dielectrophoresis (DEP) cell concentration is developed to monitor the dynamics process of ET-1 induced cardiomyocyte hypertrophy. This device can increase the sensitivity of the impedance system and also has the potential to reduce the time for detection by a significant factor.

INTRODUCTION

Cardiac hypertrophy is an established and independent risk factor for the development of heart failure and sudden cardiac death [2] [3]. At the level of individual cardiac myocytes, the cell morphology alters (increase in cell size and myofibrillar re-organization) and protein synthesis is activated [4] [5]. Since cardiac hypertrophy plays such a crucial role in many diseases such as myocardial infarction and arrhythmia, a new bio-analytical assay is expected to monitor hypertrophy in cultured cardiac myocytes non-invasively and in real time.

Using microfluidic chip to perform cellular studies on cardiac myocytes is currently a major interest due to its desirable characteristics. Werdich *et al*. reported a method to study single cardiac myocytes by recording the extracellular potential using planar microelectrodes in sub-nanoliter volume [6]. Klauke *et al*. developed a PDMS microfluidic chip to study the contraction of a single cardiomyocyte by electrical stimulation [7].

In this paper, a new cardiomyocyte-based impedance sensing system with the assistance of dielectrophoresis (DEP) cell concentration is developed to monitor the dynamic process of Endothelin-1 (ET-1) induced cardiomyocyte hypertrophy. A DEP microfluidic device is fabricated capable of concentrating cells from a dilute sample to form the cell monolayer with tight junctions. This device can increase the sensitivity of the impedance system and also has the potential to reduce the time for detection by a significant factor. Studies herein are carried out to examine the feasibility of this impedance sensing system for ET-1 induced cardiac myocyte hypertrophy. An equivalent circuit model is introduced to fit the impedance spectrum to fully understand the impedance sensing system.

DESIGN AND FABRICATION

In this task, glass microscope slides (2 in × 3 in, Fisher Scientific) were covered with a layer of positive photoresist (AZ 5214) by means of a spin-coater with 4000 rpm; the resist was then hardened at 100°C for 6 min on a hot plate. After baking, the photoresist was patterned with a mask aligner using an exposure dose of 10 mW for 20s, and subsequently developed by AZ 400 MIF developer (1:3) for 35s. After this step, the glass slides were cleaned by DI water and dried by nitrogen. The first metal layer for the DEP electrodes was deposited by sputtering of aluminum and patterned by lift-off to create the DEP electrodes. Then a thick layer of silicon dioxide was deposited by plasma enhanced chemical vapor deposition (PECVD) to completely isolate the DEP electrodes. Windows were defined in the PECVD oxide layer using conventional lithography and opened by reactive-ion-etching to access the bond pads on the first metal layer. Subsequently, the second metal layer, which serves as measurement electrodes, was deposited by sputtering 800 Å over a chromium adhesion layer and patterned by lift-off. The glass slide with microelectrodes was sealed with a custom made silicone chamber which has inlet and outlet tubes. An HP 33120A arbitrary waveform generator was used as the ac signal source to produce sinusoidal signal with a frequency specified at 2 MHz. Images were collected using a Nikon microscope with and without fluorescence filters and recorded by a charge-coupled device (CCD) digital camera (Pixera Penguin 600 CL, Pixera Corp., Los Gatos, CA). Figure 1 shows the cross section of the packaged device and the bioprocessor.

EXPERIMENTAL DETAILS

All fluids were injected into the chips using motor-driven micropump. The flow rate in the main channel of the bioprocessor was controlled by adjusting the pressure applied at the injector. The flow rate through the incubation chamber was controlled by applying a back-pressure at the chamber output using pressurized nitrogen. In this biochip, external valves at the input and output tubes were used to isolate the chip from the injection system during incubation. While in the bioprocessor, all the microbore tubes leading into and out of the chip were manually pinched to achieve isolation.

Experiments with this biochip were performed with it mounted onto a custom-made heated platform with a built-in RTD temperature sensor. Electrical connections to the chips were established by using probes mounted on micromanipulators attached to the heated platform. Experiments with the bioprocessor were done with the chip carrier mounted on the stage of a Nikon Eclipse E600 epi-florescence microscope (Nikon USA Corp., Melville, NY). During all the experiments, a computer controlled the temperature of the chips to be within ±0.2°C or better using the readings from the off RTD sensors, depending on the chip being used, to adjust the power delivered to the heaters on which the chips were mounted. The chip stages were completely enclosed to keep air drafts from disturbing the temperature.

The impedance of the electrodes in the chip was measured with an Agilent 4284A LCR meter (Agilent Technologies Inc., CA) connected to the chip through an Agilent 16048A BNC test fixture. All of these instruments were connected to a computer through a GPIB interface. The impedance measurement process was controlled by Labview (National Instruments Corp.) virtual instruments. The impedance of interdigitated electrodes was measured at 52 frequencies logarithmically spaced between 100 Hz and 1 MHz. with a 50-mV voltage excitation. Sinusoidal DEP signals were generated by an Agilent 33120A function generator.

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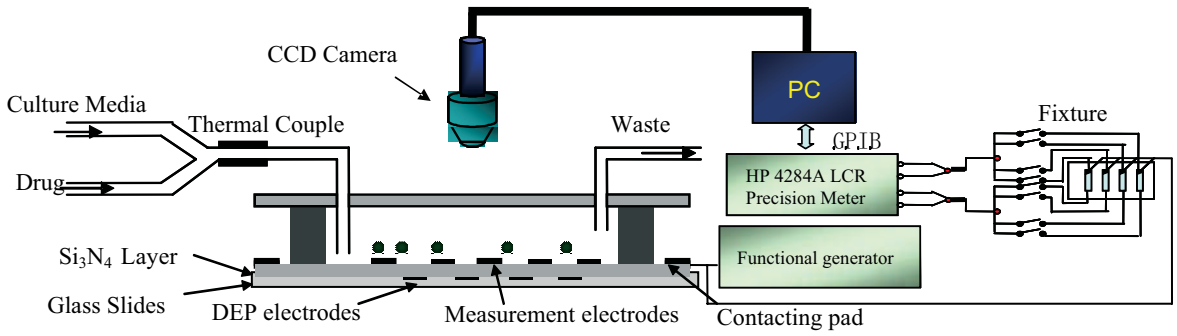

Figure 1. *Simplified cross section of impedance based biosensor system with dielectrophoresis concentration.*

All the experiments were performed with the bioprocessor heated to (37±0.1) °C. The cells suspended in PC-1 serum free media were injected at an input flow rate of ~ 1.4 µL/min, with the capture electrodes excited with a 2 V_{pp} sinusoidal signal at 2MHz (conductivity adjusted to 5 mS/m by addition of DI water). During injection, the flow rate in the incubation chamber was manually controlled to be between 4 and 10 nL/min. Flow rate control was performed manually by adjusting the pressure applied to the bioprocessor based on the observed velocity of the cells in the channels. After the whole 40 µL of sample had been forced through the bioprocessor the flow was stopped, the DEP capture electrodes were turned off, and a solution of DMEM-M199 (4:1) was injected at a flow rate of less than 0.5 µL/min. As the meida in the chamber was replaced by DMEM-M199 (4:1), the excitation voltage on the capture electrodes was increased to 3.5 V_{pp} and the frequency to 3MHz to maximize the DEP forces acting on the cells. After pinching the tubes, the impedance measurement process was started and the cells were incubated for a minimum of 24h. When a reference measurement was desired, cells were injected only into one of the two devices in a bioprocessor, where the other device received DMEM-M199 (4:1) only. The device with DMEM-M199 (4:1) provided baseline impedance in the absence of any metabolic activity.

RESULTS AND DISCUSSION

The impedance spectrum of this system can be represented by an equivalent circuit shown in Fig. 2. In this circuit, the resistor R_s is mainly due to the conductivity of the bulk solution and the wire connection whereas the Z_{CPE} represents the dielectric properties of the electrode/electrolyte and surface morphological information. R and C are the resistance and the capacitance of the layer of the absorbed proteins. R_c and C_c are elements related to the cell layer. The model presented here is derived from a physical analysis of the simplest electrochemical processes taking place at the interface and is thus bound to better represent the phenomena being measured [7]. In addition, fitting the model to impedance values at a large number of frequencies over a large range makes the measurement more sensitive. Such fitting essentially concentrates small changes in the impedance at each frequency point into larger changes in a small number of parameters that might have some physical relevance.

The impedance spectra derived from equivalent circuits composed of ideal elements, like capacitors and resistors, do not fit the measured spectra in a satisfactory way because of the inhomogeneity of the surface. To overcome this problem, it is possible to replace the ideal elements by constant-phase elements (CPEs) Z_{CPE} which account for the nonlinearities and the frequency of the elements. This is the simplest model that would properly fit the measured data over the whole frequency range and at all times during incubation.

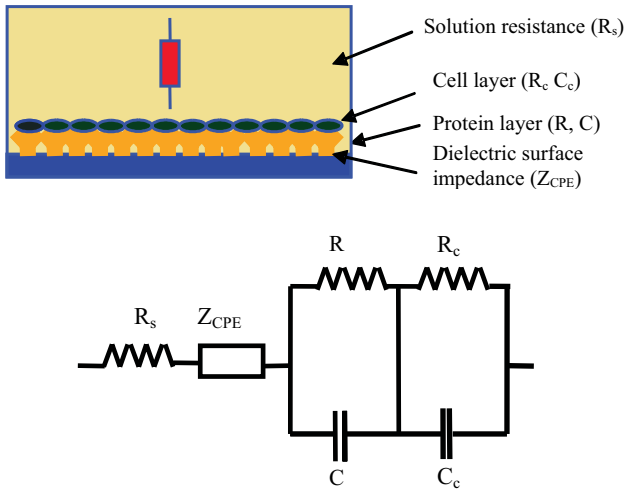

Figure 2*. Equivalent circuit for analysis. R_s is mainly due to the conductivity of the bulk solution and the wire connection whereas Z_{CPE} represents dielectric properties of the electrode/electrolyte and surface morphological information. R and C are the resistor and the capacity of the layer of the absorbed proteins. R_c and C_c are elements related to the cell layer.*

The first biochip was used to perform preliminary tests of the impedance technique with live cells incubated inside the chip. Suspensions of different concentrations of cardiac myocytes were injected into the chip and incubated in an on-chip temperature around 37°C for more than 24 hours.

Figure 3 compares the first and last impedance spectrum recorded in this experiment in two-dimensional representation. The open circle (o) represents the frequency dependant impedance spectrum before the cardiomyocyte monolayer was exposed to 100 nM ET-1 addition (t= 0h) whereas the square (□) corresponds to the impedance spectrum of the same electrode after the cells were exposed to the 100 nM ET-1 and experienced the ET-1 induced cell morphology change (t= 4h). As apparent from Fig. 2 the difference in the total impedance between vital and hypertrophy cells passes a maximum at roughly 4 kHz indicating that impedance readings at this frequency are most sensitive to the associated changes in cell morphology.

Figure 4 shows the change in impedance over time using DEP concentration method for the two cardiomyocyte cases: 4.6×10^3 cfu/mL and 5.3×10^4 cfu/mL. It clearly shows that DEP method dramatically increases the impedance amplitude which is related to the increased cell numbers on the impedance microelectrodes.

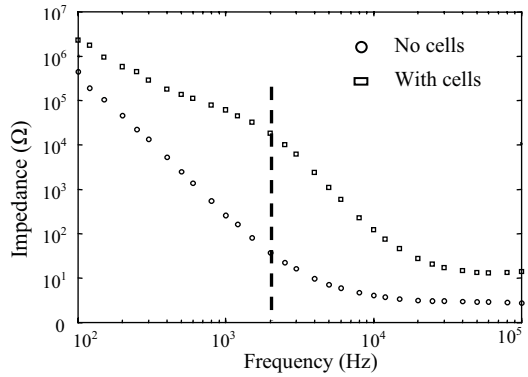

Figure 3. *The first and last impedance spectrum recorded in this experiment in two-dimensional representation.*

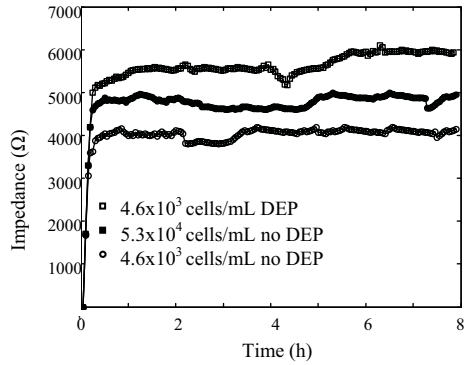

Figure 4. *DEP assisted cell concentration. As can be seen, DEP method dramatically increases the impedance amplitude.*

To assess the effects of ET-1 on the extent of cell-substrate adhesion, we monitored changes in cell-substrate resistance after the addition of ET-1. Typical results are shown in Fig. 5a. The extent of cell-substrate adhesion is expressed as "normalized" resistance, which was defined as a ratio of the resistance to the initial value before addition of ET-1. The addition of 100 nM induces a rapid increase in impedance after an initial lag phase of around 0.3h. Within 3 hours the impedance magnitude approaches the saturation value. To get a quantitative measure for the dynamics of ET-1 induced changes we determined the time necessary for half-maximum reduction of the impedance at 4 kHz (t_{Z50}). The analysis shows that the mean value of 20 similar time courses is around 1.7 ± 0.2 h. ET-1 induced a significant increase in normalized resistance, indicating strengthening of the cell-substrate adhesion at 4 h after addition of ET-1 (Fig. 5b).

Since focal adhesion kinase (FAK) has been reported to play an important role in ET-induced hypertrophy such as promoting sarcomere assembly, we compared the time course of the impedance change with the time course of FAK activation upon ET-1 exposure. Fig. 5c traces the activity of FAK before and several hours after exposing the cells to 100 nM CHX. Comparing with Fig. 5a, we can see that that even very small and clearly sub-maximum levels of FAK activity is with large changes in impedance. After 2h of ET-1 exposure FAK activity amounts to less than 55% of its final value while the impedance of the cell layer at 4 kHz has already increased for more than 80% of its total change indicative of more sensitivity of our impedance assay.

The effect of DEP concentration on the impedance spectrum with the addition of ET-1 is also explored for two cases: 4.6×10^3 cfu/mL and 5.3×10^4 cfu/mL (Fig. 6). The impedance spectrum on the concentration of 5.3×10^4 cfu/mL is treated as the baseline. The sample at concentration of 5.3×10^4 cfu/mL injected without the

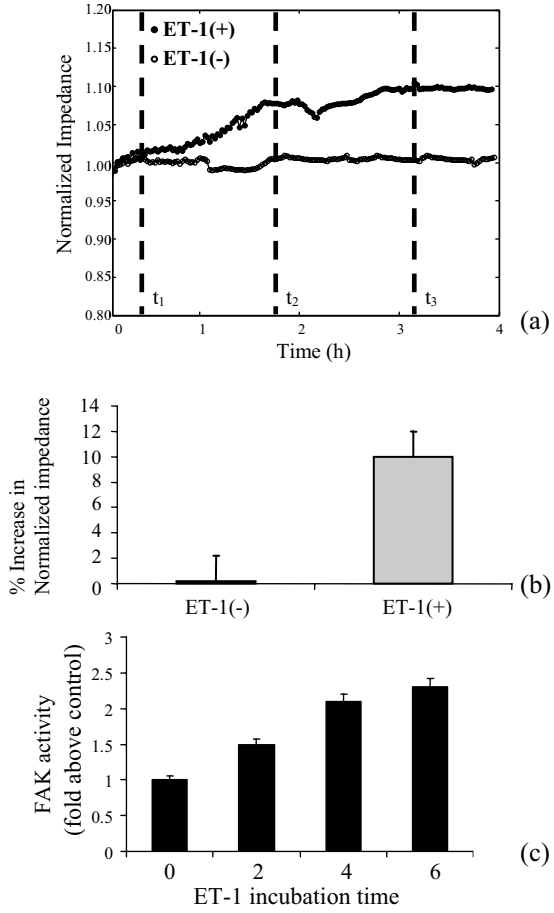

Figure 5. *Effects of ET on adhesion of cardiac myocytes to the Extracellular Matrix (ECM). After starvation for 12 hours, they were stimulated with ET-1 (nM). (a) Typical tracing representative of four replicate experiments. In all these experiments, initial resistance of cardiac myocytes was within the range of 3000 to 5000 Ω. To simplify the comparison, ordinate represents normalized. (b) Comparison of percentage increase in normalized resistance at 4 hours after addition of ET-1, ET(+), with control ET(-). Results shown are mean ± SD from four independent experiments. (c). Focal adhesion kinase (FAK) activity as function of ET-1 incubation time.*

DEP-based concentration system active increased the impedance amplitude by 10% corresponding to a cell size increase due to ET-1 addition. On the other hand, for the sample at relatively low concentration of 4.6×10^3 cfu/mL injected with the concentration system active, a very strong hypertrophy signal with the impedance amplitude increase of 18% was visible during the first hour of incubation. The DEP cell concentration method clearly increased the cell number on the impedance detection microelectrodes, the sensitivity of hypertrophy detection and made the low concentration sensing possible for very dilute cell suspensions.

To correlate this variation of impedance to cardiac myocyte behavior, the fitting procedure with the previous equivalent circuit, defined without ET-1, is applied to impedance variation as a function of ET-1 concentration. Values of R_s, Z_{CPE}, R and C remain constant in the equivalent circuit while values of R_c and C_c components vary as a function of ET-1 concentration as shown in Fig. 7. The error bars include the experimental error on impedance

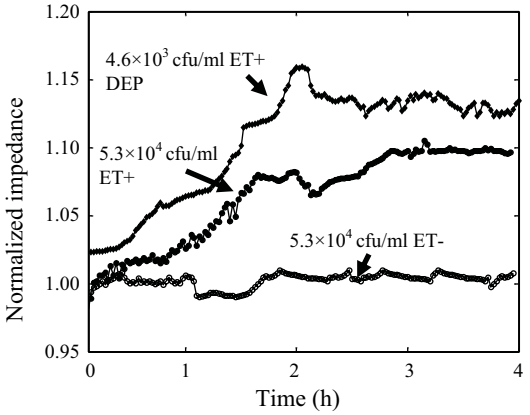

Figure 6. *Normalized impedance of cardiac myocytes injected into the bioprocessor at various concentrations, with and without the DEP-based concentration system active, plus sterile KB. Values at t=0 are defined as 100%.*

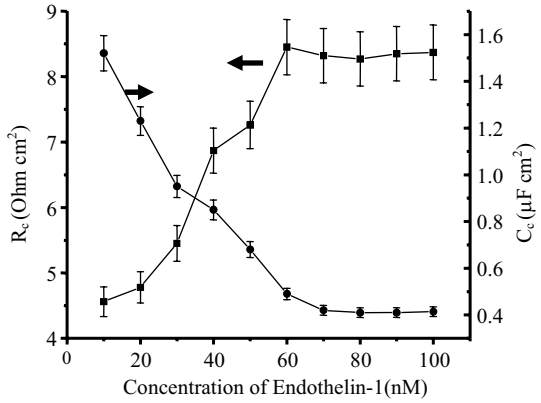

Figure 7. *Equivalent circuit parameter shows the correlation of variation of impedance to cardiomyocyte behavior. Here, R_c and C_c are parameters related to the cell layer.*

measurements and the discrepancy of experimental values related to the equivalent circuit model. As shown in Fig. 4, the cell layer resistance R_c changes from 4.52 Ω cm^2 to 8.43 Ω cm^2 due to cardiac myocyte hypertrophy, with an increase by 86.50% and the capacitance C_c change from 1.52 μF cm^2 to 0.41 μF cm^2. The general evolution of R_c and C_c is indicative of the effect of ET-1 on cardiomyocyte morphology and a saturation effect is observed for both components for concentration higher than 60 nM which corresponds to the saturation effect on the experimental curve.

CONCLUSIONS

In this paper, a new cardiaomyocyte-based impedance sensing system with the assistance of dielectrophoresis cell concentration was developed to monitor the dynamics process of Endothelin-1 induced cardiomyocyte hypertrophy. When cultured *in vitro* cardiac myocytes are up to 20×100 μm in size and can form close contacts to the substrate. Both of these characteristics are favorable for the development of high seal resistances. Thus, they make an excellent choice for impedance studies where a large cellular size compared to the extracellular electrode diameter is desirable. Furthermore, when cultured at the appropriate densities, cardiac myocytes develop cytoplasmic bridges (gap junctions) between cells to form moncellolayers in culture. These tight junctions help inhibit cell movement, thereby deceasing the mobility of cardiac myocytes *in vitro.* For this purpose, a dielectrophoresis microfluidic device is fabricated capable of concentrating cells from a dilute sample to form a cell monolayer with tight junctions. This device can increase the sensitivity of the impedance system and also has the potential to reduce the time for detection by a significant factor. Studies herein were carried out to examine the feasibility of this impedance sensing system for ET-1 induced cardiac myocyte hypertrophy. An equivalent circuit model is introduced to fit the impedance spectrum to fully understand the impedance sensing system.

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