



A novel microfluidic impedance assay for monitoring endothelin-induced cardiomyocyte hypertrophy

Mo Yang^{a,b}, Chee Chew Lim^c, Rongli Liao^d, Xin Zhang^{a,*}

^a Laboratory for Microsystems Technology, Department of Manufacturing Engineering,
15 Saint Mary's Street, Boston University, Boston, MA 02215, USA

^b Biomedical Engineering Division, Department of Health Technology and Informatics,
The Hong Kong Polytechnic University, Hong Kong, PR China

^c Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA 02118, USA

^d Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

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Abstract

Cardiac hypertrophy is an established and independent risk factor for the development of heart failure and sudden cardiac death. At the level of individual cardiac myocytes (heart muscle cells), the cell morphology alters (increase in cell size and myofibrillar re-organization) and protein synthesis is activated. In this paper, a novel cardiomyocyte-based impedance sensing system with the assistance of dielectrophoresis cell concentration is reported to monitor the dynamic process of endothelin-1-induced cardiomyocyte hypertrophy. A dielectrophoresis (DEP) microfluidic device is fabricated capable of concentrating cells from a dilute sample to form a confluent cell monolayer on the surface of microelectrodes. 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. To examine the feasibility of this impedance sensing system, cardiomyocytes are treated with endothelin-1 (ET-1), a known hypertrophic agent. ET-1 induces a continuous rise in cardiomyocyte impedance, which we interpret as strengthening of cellular attachments to the surface substrate. An equivalent circuit model is introduced to fit the impedance spectrum to fully understand the impedance sensing system.

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1. Introduction

Cardiac hypertrophy is an established and independent risk factor for the development of heart failure and sudden cardiac death (Levy et al., 1990; Ruskoaho, 1992). Cardiac hypertrophy is defined as an increase in heart size and/or myofibrillar volume without a change in myocyte number. At the level of individual cardiac myocytes, the cell morphology alters (increase in cell size and myofibrillar re-organization) and protein synthesis is activated (Lorell and Carabello, 2000; Diane et al., 2000). Currently, the cardiac myocyte hypertrophy, *in vitro*, is monitored using a combination of radiological labeling, immunofluorescence labeling and kinase activity assessment based on morphological and immunochemical characteristics. The whole

process is invasive, time-consuming and unable to monitor the whole dynamic process. Therefore, it is necessary to develop a novel bio-analytical sensor device to obviate these cumbersome experimental assays and monitor cardiac myocyte hypertrophy non-invasively and in real time.

It has long been recognized that the membrane of biological materials, such as cells, exhibits dielectric properties. This biological property is exploited by the emerging sensor technology referred to as electric cell-substrate impedance sensing (ECIS), to monitor the morphology, viability, and environmental change for the adherent cells (Giaever and Keese, 1991, 1993). The use of living cells as sensor elements provides the opportunity for high sensitivity in a broad range of biologically active substances that affect the response of the cells. In ECIS, a current is applied through cultured cells plated on gold electrodes. Upon the attachment and spreading of cells on the gold electrodes, the impedance increases because the cells act as insulating particles to restrict the flow of current. The chang-

* Corresponding author. Tel.: +1 617 358 2702; fax: +1 617 353 5548.
E-mail address: xinz@bu.edu (X. Zhang).

ing impedance can be continuously monitored and interpreted to reveal information about cell spreading and micromotion (Ehret et al., 1997; Kowolenko et al., 1990). Impedance measurements have been used to monitor the behavior of many cell types such as macrophages (Mitra et al., 1991), endothelial cells (Xiao et al., 2002; Tiruppathi et al., 1992) and fibroblasts (Luong et al., 2001). Arndt et al. (2004) used endothelial cells as the sensor elements to study the cycloheximide-induced *apoptosis*. The feasibility of glucose sensing by fibroblasts over the physiological range of glucose concentrations was also demonstrated and shown to be inhibited by addition of cytochalasin B (Tlili et al., 2003).

In this work, a new cardiomyocyte-based impedance sensing system with the assistance of dielectrophoresis cell concentration has been developed. When cultured *in vitro*, cardiac myocytes are up to $20\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ 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. To further refine our impedance sensing system, a dielectrophoresis (DEP) microfluidic device has been fabricated capable of concentrating cells from a dilute sample to form a confluent cell monolayer. 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. To test the feasibility and practicality of our impedance sensing system, we treat cardiomyocytes with endothelin-1 (ET-1), which is known to induce cardiac myocyte growth (hypertrophy). An equivalent circuit model is introduced to fit the impedance spectrum to fully understand the impedance sensing system.

2. Materials and methods

2.1. Cell isolation and culture

Left ventricular (LV) myocytes were isolated and permeabilized according to a previously described protocol (Lim et al., 2001). Male Wistar rats, weighing between 200 and 250 g, were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and heparinized (200 IU i.v.). The hearts were rapidly excised and arrested in ice-cold cardioplegic Krebs Bulbring (KB) solution (85 mmol/L KOH, 30 mmol/L KCl, 30 mmol/L KH_2PO_4 , 3 mmol/L MgSO_4 , 0.5 mmol/L EGTA, 10 mmol/L HEPES, 50 mmol/L L-glutamic acid, 20 mmol/L taurine, 10 mmol/L 2,3-

butanedione monoxime, and 10 mmol/L glucose). The hearts were cannulated and retrogradely perfused via the aortic root with 1.2 mmol/L Ca^{2+} Tyrode solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl_2 , 0.5 mmol/L MgCl_2 , 10 mmol/L HEPES, 10 mmol/L glucose, pH 7.4), followed by Ca^{2+} free Tyrode solution until the hearts ceased beating. Hearts were then perfused with a digestion solution containing 0.08% collagenase A (Boehringer) and 0.02% protease XIV (Sigma–Aldrich). All perfusion solutions were equilibrated with 100% oxygen and maintained at 37°C throughout the isolation process. The digestion solution was eventually washed out with KB solution, and the hearts removed from the cannula. The LV was separated, minced, and gently agitated allowing the myocytes to be dispersed in KB solution. The myocyte suspension was subsequently filtered through a nylon mesh, resulting in a typical yield of >90% rod-shaped cells. The isolated cells were then washed with DMEM supplemented with albumin, creatine, carnitine, and taurine (ACCT media), with the addition of ascorbic acid (100 μM) and were either directly plated onto the microchamber with the microelectrodes precoated with laminin (Becton–Dickinson) or injected into the chamber for DEP capture of myocytes (see below).

2.2. Design and fabrication

In this task, glass microscope slides (2 in. $\times$ 3 in., Fisher Scientific) were covered with a layer of positive photoresist (AZ 5214) at 4000 rpm by means of a spin-coater; 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 20 s, and subsequently developed by AZ 400 MIF developer (1:3) for 35 s. 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 liftoff 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, a gold layer over a chromium adhesion layer was deposited by sputtering and patterned by lift off methods to create the measurement electrodes. The glass slide with microelectrodes was sealed with a custom-made silicone chamber with inlet and outlet tubes. An HP 33120A arbitrary waveform gener-

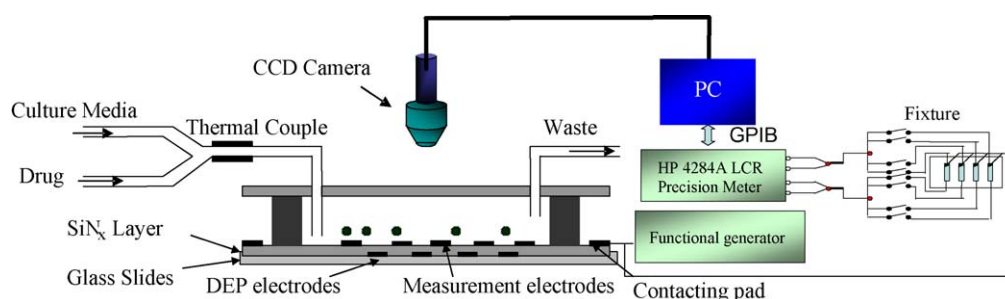


Fig. 1. Simplified crosssection of experimental set-up.

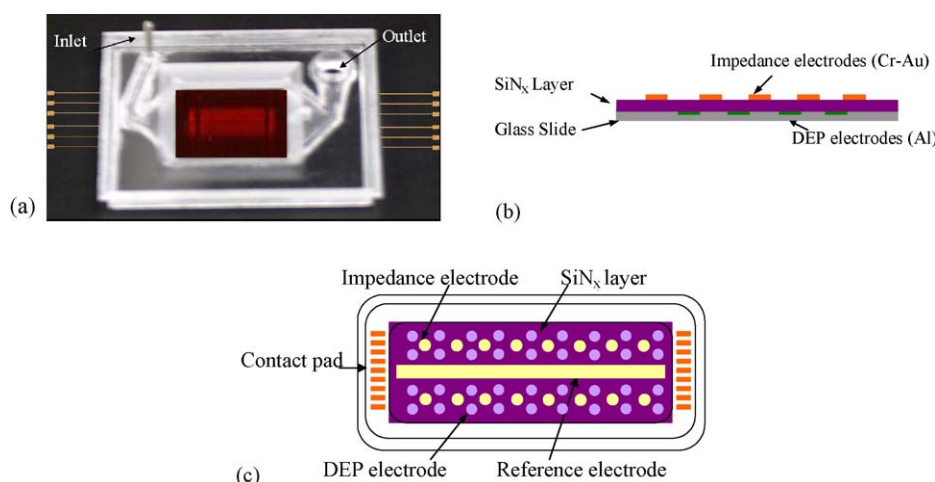


Fig. 2. (a) Photograph of device with flow chamber; (b) scheme of chip layer; (c) scheme of chip top view.

ator 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 and recorded by a charge-coupled device (CCD) digital camera.

2.3. Experimental set-up

The scheme of the experimental set-up for dielectrophoresis concentration and impedance measurements is presented in Fig. 1. A polycarbonate flow perfusion chamber with an internal volume of 30 μL sealed onto the biochip was used for all measurements (Fig. 2a). Schematic cross-section of chip layers and top view of the biochip are presented in Fig. 2b and c. DEP electrodes were used to concentrate cells from a dilute sample to form the cell monolayer and impedance electrodes (Cr–Au) were used to monitor the cardiomyocyte hypertrophy after the cell monolayer formation. Myocytes were injected into the chamber at a constant rate from one end using a motor driven micropump, and superfused with modified DMEM buffer solution. The flow rate in the microfluidic channel of the biochip was controlled by adjusting the pressure drop between the inlet and the outlet through a microflow controller (Cell Micro Controls Ltd., VA).

Experiments with the chip assembly were performed inside a custom-made heated platform with a built-in RTD temperature sensor. The chip assembly was completely enclosed to prevent the air flow disturbance. A temperature control system (Cell Micro Controls Ltd., VA) linked to the temperature sensor was used to keep a constant temperature of 37 $^{\circ}\text{C}$. The carbon dioxide concentration inside the chamber was maintained at 5% and humidified to prevent excessive evaporation of the medium. Electrical connections to the chips were established by using probes mounted on micromanipulators attached to the heated platform. This chamber with all of its components was capable of ensuring cell viability over long periods of time and stabilizing cell physiology in the absence of chemical agents.

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., Austin, TX) 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.

2.4. On-chip cell capture

All the experiments were performed with the bioprocessor heated to $37 \pm 0.2^{\circ}\text{C}$. The cells suspended in DMEM media were injected at an input flow rate of approximately 1.4 $\mu\text{L}/\text{min}$. The capture electrodes were excited with a 2 V_{pp} sinusoidal signal at 2 MHz. During injection, the flow rate in the incubation chamber was manually controlled to be between 4 to 10 $\mu\text{L}/\text{min}$. Flow rate control was performed using a flow controller based on the observed velocity of the cells in the channels. After the whole sample had been forced through the bioprocessor, the flow was stopped and the DEP capture electrodes were turned off. After pinching the tubes, the cells were incubated for a minimum of 24 h to form a confluent layer before the impedance measurement was started. To get the baseline impedance, a reference measurement was desired. Cells were injected into one device in a bioprocessor, where the other device received DMEM only. The device with DMEM media provided baseline impedance in the absence of endothelin-1.

3. Results and discussion

In this work, we have developed a novel bio-analytical system which can monitor cardiomyocyte hypertrophy in monolayers of adherent cells. This biosensor also provides convenient methods for cell concentration and patterning, which reduce the detection time and increase the sensitivity. The majority of existing techniques to monitor cardiac myocyte hypertrophy, *in vitro*, measure changes in (1) cellular protein content (Axion Exhibit 1022), (2) radio-isotope labeled amino acids, (3) cardiomyocyte

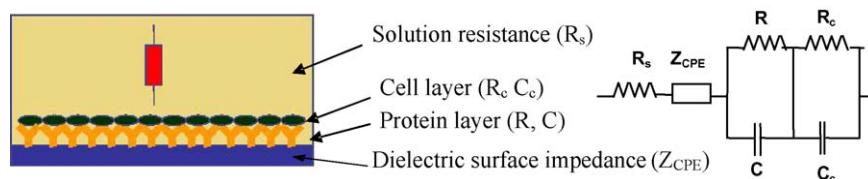


Fig. 3. Equivalent circuit for analysis. R_s is mainly due to the conductivity of the bulk solution and the wire connection, whereas the Z_{CPE} represents the dielectric properties of the electrode/electrolyte and surface morphological information. R and C are the resistance and the capacitance of the layer of the absorbed proteins. R_c and C_c are elements related to the cell layer.

morphology (by immunofluorescence labeling of myofilament proteins), or (3) kinase activity (such as focal adhesion kinase, FAK). The described cardiac myocyte impedance sensing system obviates these cumbersome experimental assays and provides a convenient and rapid *in vitro* method to measure cardiac myocyte hypertrophy.

3.1. The equivalent circuit of the impedance sensor

The impedance spectrum of this system can be represented by an equivalent circuit shown in Fig. 3. 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 overall impedance spectrum signal can be fitted by a mathematical model that was recently derived by Tlili et al. (2003) based on the electrochemical processes taking place at the interface. Fitting the model to impedance values in frequency spectrum over a large range makes the measurement more sensitive, i.e. an optimistic frequency can be chosen for the most sensitive impedance readings. Such fitted circuit model parameters essentially concentrates small changes of the impedance amplitude at each frequency point into larger changes which can be promising indicators with biophysical 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} (MacDonald, 1987; Omanovic and Metikos-Hukovic, 1995) which account for the non-linearities and the frequency of the elements by expressing the electrical impedance in terms:

$$Z_{CPE} = \frac{1}{Q_{CPE}(j\omega)^n}$$

where $j = \sqrt{-1}$. This simplest model can properly fit the measured data in the whole frequency spectrum and at all time points during incubation (Gomez-Sjoberg et al., 2005).

3.2. Characterization of the impedance sensor

The 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 h.

Fig. 4 compares the first and last impedance spectrum recorded in this experiment in two-dimensional representation. The open circle (○) represents the frequency dependent impedance spectrum before the cardiomyocyte monolayer was exposed to 100 nM endothelin-1 (ET-1) addition ($t=0$ h in Fig. 5), 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 ($t=4$ h in Fig. 5). As apparent from Fig. 4, the difference of total impedance amplitude between vital and hypertrophy cells passes a maximum at roughly 2 kHz indicating that impedance readings at this frequency are most sensitive to the associated changes in cell morphology.

3.3. On-chip detection of ET-induced cardiomyocyte hypertrophy

Since cardiac myocytes are a relatively high motility cell type, it is possible that the effects observed with the chemical agents are covered by cell motility rather than biochemical modulation. The inhibition of this motion is therefore required if biochemical modulation is to be confirmed. Cytochalasin-D has been used to successfully inhibit movement of cardiac myocytes, however, this compound (when used at high enough concentrations to inhibit movement) causes the cells to detach from the surface, resulting in a reduction of the measured impedance (Lo et al., 1993). Glucose deprivation has been shown to be a good way to reduce impedance fluctuations for confluent cells in monolayers over extended periods of time (Lo et al., 1993). Thus, in our

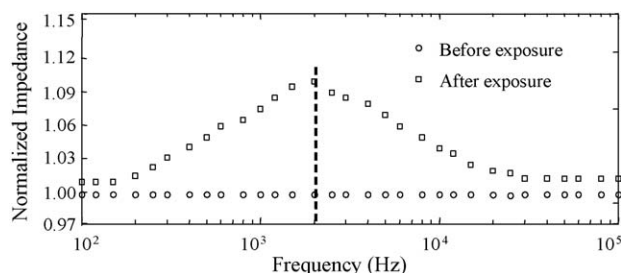


Fig. 4. Frequency spectrum of electrical impedance of gold-film electrodes covered with a confluent of cardiac myocyte cells before (○) and after (□) exposure to 100 nM endothelin-1 (ET-1) after 5 h. The dotted line indicates impedance values at a frequency of 2 kHz. The normalized impedance is defined as Z_{ET-1}/Z_{ET-} . Z_{ET-} is the impedance before addition of ET-1.

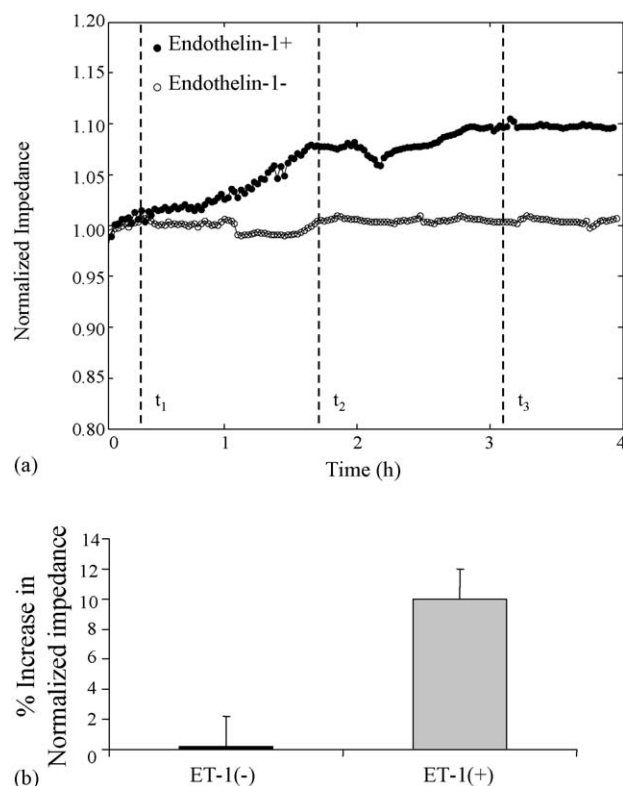


Fig. 5. Effects of ET on adhesion of cardiac myocytes to the extracellular matrix (ECM). Cardiac myocytes were cultured on the surface of laminin-coated wells manufactured especially for measurement of resistance. After glucose deprivation for 12 h, they were stimulated with ET-1 (endothelin-1) (nM). (a) Typical tracing representative of four replicate experiments. In all these experiments, initial resistance of cardiac myocytes was within the range of 3000–5000 Ω . To simplify the comparison, ordinate represents normalized impedance. (b) Comparison of percentage increase in normalized impedance at 4 h after addition of ET-1, ET(+), with control ET(–). Results shown are mean $\pm$ S.D. from four independent experiments.

experiment, the first impedance measurement was done about 12 h after the last media change of DMEM in incubation to decrease cell motility.

To assess the effects of ET-1 on the extent of cell-substrate adhesion, the changes in cell-substrate resistance were monitored after the addition of ET-1. We first monitored impedance values at frequency 2 KHz where a confluent monolayer of cardiomyocytes was exposed 100 nM ET-1. The extent of cell-substrate adhesion was expressed as “normalized” resistance, which was defined as a ratio of the resistance (Z_{ET+}) to the initial value before addition of ET-1 (Z_{ET-}). The addition of 100 nM ET-1-induced a rapid increase in impedance after an initial lag phase of around 0.3 h (Fig. 5a). Within 3 h the impedance magnitude approached 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 2 kHz (t_{Z50}). The analysis showed that the mean value of 20 similar time courses was 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).

3.4. Cardiomyocyte hypertrophy detection with DEP concentration

To test the complete process of cell concentration and hypertrophy detection, cardiomyocytes, suspended in DMEM at concentrations of 4.6×10^3 and 5.3×10^4 mL^{-1} , were injected into the chip at a flow rate of approximately 1.4 $\mu\text{L}/\text{min}$ and incubated for at least 24 h at 37 $^{\circ}\text{C}$. To achieve a highly efficient concentration and capture process, the DEP forces in the capture stages of the bioprocessor had to be strong enough to counteract the drag forces exerted on the cells by the fluid around them. The DEP forces on cardiomyocytes suspended in DMEM were too weak to achieve an efficient concentration process at reasonable flow rates. However, cardiomyocytes experienced strong DEP forces at an excitation frequency of 2 MHz and the amplitude of 2 V_{pp} . With the right combination of medium conductivity, flow velocities, and DEP excitation voltage and frequency, the concentration efficiency was virtually 100%.

During injection of the sample with 5.3×10^4 mL^{-1} , the DEP electrodes were off so that the cells would not become concentrated in the incubation chamber. The other sample 4.6×10^3 mL^{-1} was injected with the DEP electrodes activated, which caused most of the cells in the samples to accumulate on the surface of microelectrodes. The actual number of cells collected was not known, but it was visually confirmed that only a very small fraction of the cells escaped the concentration process (no more than 5%). The frequencies chosen for plotting each curve, out of the 51 frequencies that were monitored, were those for which the relative impedance change was the largest at the frequency 2 kHz. Fig. 6a shows the effect of DEP concentration on the impedance spectrum with the addition of ET-1. In the absence of ET-1, the impedance spectrum of the concentration of 5.3×10^4 mL^{-1} did not change, as previously shown (Fig. 5a). The sample at concentration of 5.3×10^4 mL^{-1} injected without the DEP-based system active increased the impedance amplitude by 10% due to ET-1 addition. On the other hand, for the sample at relatively low concentration of 4.6×10^3 mL^{-1} injected with the DEP system active, an ET-1-induced increase in impedance amplitude of 18% was detectable in the impedance spectrum. These data demonstrated the utility of the DEP cell concentration method, as the ability to concentrate cells from a dilute sample on the impedance detection microelectrodes would further enhance the sensitivity of our biosensor.

3.5. Equivalent circuit parameter fitting

To correlate the variation of impedance to cardiomyocyte 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. 6b. The error bars include the experimental error on impedance 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 to 8.43 Ω due to cardiac myocyte hypertrophy, with an increase by 86.30% and

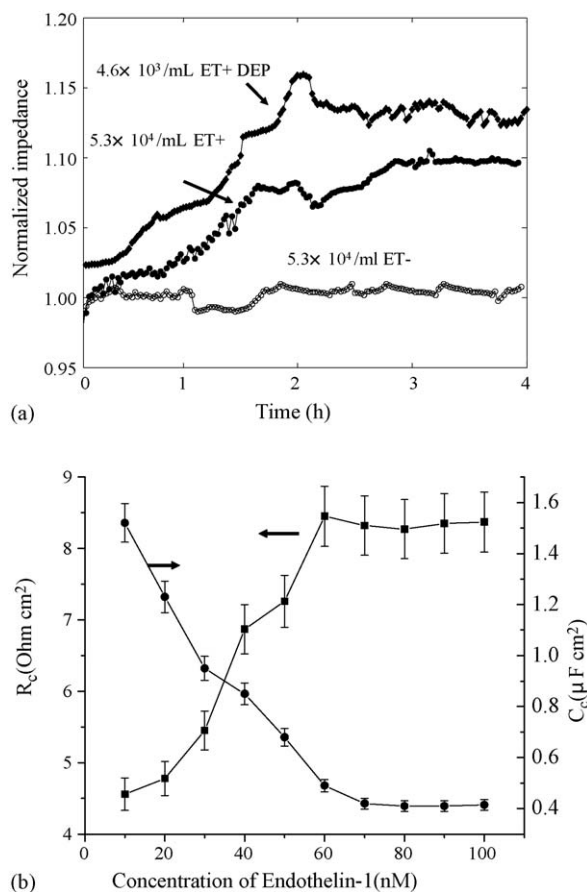


Fig. 6. (a) Normalized impedance of cardiac myocytes injected into the bioprocessor at various concentrations, with and without the DEP-based concentration system active. Values at $t=0$ are defined as 100%. (b) Impedance spectrum of electrodes with a confluent monolayer of cardiac myocytes for circuit model parameters R_c and C_c .

the capacitance C_c changes from 1.52 to 0.41 $\mu\text{F cm}^2$. Since R_c and C_c are elements related to cell layer (Fig. 3), the general evolution of R_c and C_c is indicative of the effect of ET-1 on cardiomyocyte morphology. A saturation effect is observed for both components for ET-1 concentrations higher than 60 nM which corresponds to the saturation effect on the experimental curve. The increase of R_c is related to the cell-substrate adhesion enhancement which can be used to study focal adhesion kinase (FAK) activity and the increase of C_c is more related to the cell size and cell-electrode gap change during the cardiomyocyte hypertrophy dynamic process. Combining with the immunochemical methods, the fitted circuit model parameters of

the impedance spectrum can be promising indicators to explore the various effects of ET-1 on the cardiomyocyte hypertrophy.

4. Summary and conclusions

In this work, a new cardiomyocyte-based impedance sensing system with the assistance of DEP cell concentration has been developed to monitor the dynamic process of endothelin-1-induced cardiomyocyte hypertrophy. The systems are fabricated on glass substrates utilizing microfabrication technologies. By integrating the DEP cell concentration system with the impedance sensing device, we are able to significantly increase the sensitivity of the impedance system, which reduces the time for detection by a significant factor. This novel and relatively simple biosensor has the potential to become a valuable high-throughput experimental approach in studying *in vitro* cardiomyocyte hypertrophy research.

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