

CARDIAC TOXICITY SCREENING USING POLYMERIC CANTILEVER INTEGRATED WITH CELL STIMULATORS

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ABSTRACT

This paper describes a smart cantilever device to precisely monitor the drug-induced contraction forces of cardiac muscle cells. The cantilever device is composed of two parallel microelectrodes and 3D micro-patterns on the surface. The microelectrodes are utilized to synchronize beating of individual cell during drug screening experiments. These also enhance the expression of connexin 43 (Cx-43) of gap junctions that electrically couple cardiac cells. The optimized 3D microstructures greatly improve the contraction force. These physical stimulations (electrical and mechanical) are very effective to mature cardiac cells obtained from neonatal rats. The fabricated cantilever devices are also employed for pre-screening of drugs in cardiac toxicity.

INTRODUCTION

Heart failure is one of the urgent and global problems not only in the developed countries. Statistical information of the American Heart Association showed 17.3 million people died due to the heart disease and stroke in the last year. The report expected that the number of patients will increase more than 23.6 million by 2030 [1]. Hereof, adverse effect of drug reactions was the leading cause of the death [2]. In this regard, new solutions are needed to regenerate the cardiac tissue and to develop easier method for drug screening.

Electrical stimulation is one of the influential methods for exploring and manipulating cardiac cell behavior [3]. It has been widely used for more than a century in laboratories and clinics both for research and therapies [4]. By applying an electric field to the cell, it can generate an action potential, which propagates to the coupled cell via gap junction. There have been many efforts to investigate the effect of alternating current (AC) stimulations on skeletal muscle cell [5], neurons [6], and cardiomyocytes (CMs) derived from rat primary CMs [7] and iPSC [8]. In terms of electrical stimulation on cultured cardiac cells, *in vitro* models for detecting arrhythmia [9] and mechanoelectrical feedback [10] for contraction and relaxation phenomenon have been developed. On the other hand, synchronous contracted CMs could be helpful in establishing physiological structure and function.

The electrical stimulating system for CM ion channel expression allows us to more deeply understand cell growth and working function. There have been several works to develop bioreactors by using electrical conductive nanofibers [11], interdigitated ITO electrodes [12], and point-stimulation electrode [13] for studying cardiac muscle cells. However, the previously reported devices focused only on an electrical assay of CMs without including a mechanical aspect.

Another issue for *in vitro* culture of CMs is to implement a 3D environment, which can mimic the

mechanical stimulation in the heart. Previous studies have attempted to provide grooved surfaces for CMs to mimic *in vivo* 3D circumstances, and have shown that the alignment, orientation and shape of the cells were guided by the grooved surfaces. They developed micro cantilevers to validate and measure the changes of the contraction force of the CMs responding to the topology of cell adhesion surfaces. Those studies concerning the 3D organization of CMs aimed to obtain a more complete understanding toward cardiac tissue engineering.

In this study, we have focused on a direct conductive electrical stimulator together with mechanical stimulus for immature cell growing function and drug toxicity screening. The fabricated electrode array is individually positioned to provide a spatially varying electrical field to apply cell growing function.

MATERIAL AND METHOD

Design and modeling stimulator

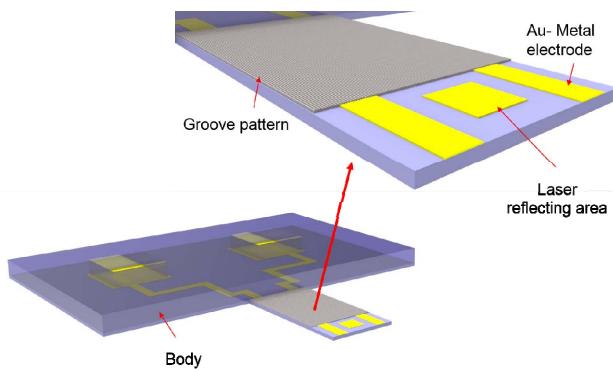


Figure 1: The designed stimulator based on biocompatible photosensitive polymer cantilever

As shown in Fig. 1, the parallel type electrode array is proposed to produce an electric field for the stimulation of primary ventricular cells. Fig. 2 shows the fabrication process flow of the current photosensitive polymer cantilever. A four inch single polished silicon wafer was used as a starting substrate. Then, a 300 nm-thick SiO₂ layer as a sacrificial layer to separate the polymer cantilever from the silicon wafer was grown by the wet oxidation. To produce the cantilever layer of 16 μm thick, a negative photoresist was spin-coated. In order to avoid surface stress, we chose a single polymer layer. Then, a 100 nm-thick Au electrodes were fabricated by e-beam evaporation and subsequent etching with the masking pattern of AZ 5214 positive photoresist. To form the thick cantilever body structure (thickness of ~120 μm), a photolithography using the higher viscous photosensitive polymer was carried out. Finally, the sacrificial layer (SiO₂) was removed in (1:6) BHF solution for several hours. To make a highly cross-linked stable surface, the

additional post-process was used. In brief, the flood expose (total exposure energy of 6000 mJ/cm²), hard baking at 95 °C for four hours, and sterilization in the autoclave was performed.

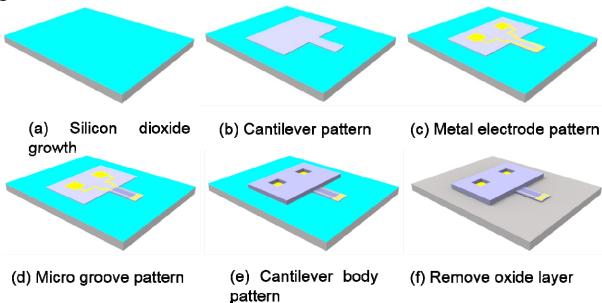


Figure 2: Stimulator fabrication process flow, (a) nearly 300 nm silicon dioxide growth for sacrificial layer, (b) photosensitive highly adhesive negative photoresist with 16 μ m thickness for cantilever pattern, (c) 100 nm Au metal electrode pattern, (d) 3 μ m pitch distance groove pattern, (e) photosensitive high viscosity negative photoresist for cantilever body, (f) etching out silicon dioxide by BHF for releasing cantilever from silicon substrate.

Isolation of neonatal rat ventricular myocyte (NRVM)

CMs were isolated from 1-3 day old neonatal rat heart, which was approved by the Animal Ethics Committee of Chonnam National University. In brief, neonatal rats were decapitated, and then blood transfused from a decapitated area. The tiny heart was quickly isolated, washed in enzyme solution, cut into small pieces and dissolved in the enzyme substance until the tissue disintegrated. The cell suspension solution was incubated at 37°C with 5% CO₂ humidity. The used culture medium was a plating medium supplemented with 10% fetal bovine serum (FBS). When the CMs were seeded on the nontoxic polymer cantilever surface, fibronectin was used as an extra cellular matrix (ECM). The cardiac cells were cultured on the stimulator at a density of 1000 cell per mm². The factors that influence cell culturing are the composition and formation of the media, the CO₂ supply, the culture temperature, etc. These factors were controlled via the interaction between the cells and the substrates, the solubility, etc.; controlling these factors influences the cell growth. The culture fluid was made from DMEM 67% (Dulbecco's modified Eagle's medium, LONZA), M199 17% (heparin sodium salt from porcine intestinal mucosa, Sigma-Aldrich), horse serum 10% (Sigma-Aldrich), FBS 5% (supplemented with 5% fetal bovine serum, Sigma-Aldrich), and penicillin-streptomycin 1% (Sigma-Aldrich). In the culture fluid (DMEM), in addition to the carbon source, energy source, nitrogen source, inorganic salt, and trace elements, there is a buffering agent included. FBS includes an element for promoting cell growth and activity. Additionally, 1% penicillin-streptomycin was used as an antibiotic.

Measurement system and electrical stimulation

Fibronectin (Corning) solution with a concentration of a 50 μ g/mL was coated on the surface of polymeric cantilevers for 45 minutes at room temperature and cardiac primary cells were then seeded on the fibronectin layer.

After finishing pre-culturing, the electrical stimulation using square monophasic pulses was applied from 4 days. Various stimulation voltages, frequencies and pulse durations were tested to define the optimal electrical stimulation parameters. Initially the electrical stimulation frequency ranged from 0.25 Hz to 3 Hz was tested. From the experimental result (data not shown here), the cantilever displacement was found to decreased when the applied frequency was changed. When the frequency was increased above 2 Hz, the cantilever could not restore its initial position. In this regard, the frequency of 0.5 Hz was selected for the electrical stimulation. The optimal parameters for the electrical stimulation were determined as electric field of 1.66 V/mm, frequency of 0.5 Hz and duration time of 2 ms. Under these conditions, the stable electrical stimulation inside the cell culture medium could be obtained. In this experiment we have changed working solution every 3 day to avoid any contamination. During the electrical stimulation, we also simultaneously measure the displacement of the polymer cantilever using a laser vibrometer-based measurement system. Through the measured displacement, the contraction force of CMs cultivated on the polymer cantilever could be evaluated. The used measurement system also included the function generator to modulate amplitude and frequency of the input signal and electrical circuit to capture the output signal from the device.

Immunofluorescence

To stain adhesion sites and parts of the cytoskeleton, cells were fixed 3.7% formaldehyde dissolved in BSA (1%) buffer for 20 min at room temperature, followed by rinsing step in DPBS with 3 times. Cells were permeabilized in 0.1% Triton X-100 in BSA for 5 min and blocked in 10% normal goat serum for an additional 30 min. All antibodies were diluted in blocking solution. Antibody incubations were performed at room temperature for 90 min, then washing steps between the primary and secondary antibodies. Samples were mounted in Gel-Mount (Biomedica, Foster City, CA) containing 50 mg/ml 1, 4-diazabicyclo octane (Sigma). We used as primary antibodies a mouse monoclonal connexin 43 (Cx 43, Sigma). The secondary antibody was a phalloidin F-actin fragment against mouse IgGs produced in goat and conjugated to Cy3 (Dianova, Hamburg, Germany). To visualize filamentous actin, we used phalloidin coupled to fluorescein (Sigma).

EXPERIMENT AND RESULTS

Fig. 3(a) shows an optical image of the fabricated cantilever-shaped stimulator. The dimension of the cantilever was 4000 μ m in length, 2000 μ m in width, and 16 μ m in thickness, resulting in the theoretical spring constant of 0.064 N/m. The cantilever body had two square pads of 2 mm \times 2 mm each for a wire bonding. One end of a wire was connected to the electrode pad on the cantilever body, while the other end of the wire was connected to the electrical circuit which generated the signal for electrical stimulation. Cell cultures were performed on the polymer cantilever with the patterned electrode with a width 300 μ m, length 4000 μ m and in 1000 μ m spacing (Fig. 3(b)). The size of micro groove patterns was optimized with

aligning CMs to get higher displacements of the cantilever. Fig. 3(c) shows an SEM image of the fabricated groove pattern with 3 μm spacing and 0.8 to 1.3 μm depth.

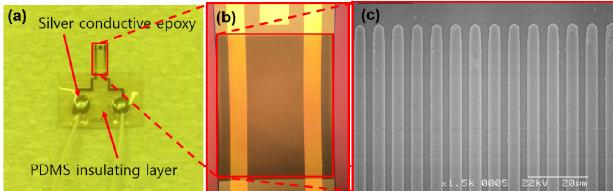


Figure 3: Optical and SEM image of polymer cantilever. (a) optical image of cantilever top view with wire bonding, (b) optical images of cantilever working area with electrode pattern, (c) SEM image of 3 μm pitch distance groove pattern.

Optical microscopic images of CMs growing on the different surfaces are shown in Figs. 4(a) and 4(b). The CMs on the flat cantilever surface were randomly aligned whereas the CMs on micro groove patterned surface were aligned along the groove. To measure the contraction force of cardiac primary cells cultured on the biocompatible polymer stimulator, a laser vibrometer-based measuring system was utilized to measure the vertical displacement with high accuracy. The measurement system was controlled by Lab-VIEW program to get input and output signals simultaneously. Aligned cardiac primary cells on the groove cantilever produced higher contraction forces as compared with those on flat cantilever.

The displacement of the cantilever was found to be linearly increased in the early culture time. The maximum displacement of both the two surfaces were observed at 10 day. The highest displacement $8 \pm 0.1 \mu\text{m}$ and $25 \pm 0.6 \mu\text{m}$ for flat and groove cantilevers respectively. After 10 day, the cantilever displacement was decreased dramatically due to the finite *in vitro* life time of CMs. The cantilever displacement was converted to the contraction force using the spring constant of cantilever (0.064 N/m). The contraction forces in the absence and presence of micro grooves on the cantilever were $0.5 \pm 0.006 \mu\text{N}$ and $1.6 \pm 0.03 \mu\text{N}$, respectively. All data were expressed as mean \pm SEM. Calculations were performed using OriginPro 8 software (Origin Lab Corporation, Northampton, MA).

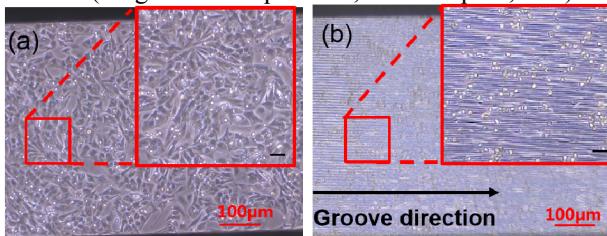


Figure 4: Primary cardiac cells generated vertical displacements on the different topographical surfaces of the cantilever. (a) and (b) cardiac cells growing in the absence and presence micro grooves groove patterned surface.

The L-type Ca^{2+} channel blocker that is a class IV anti-arrhythmia agent, verapamil, decreases the contraction force as a function of the drug concentration. To evaluate the toxicity of drugs treated to cardiac primary cells, verapamil was diluted in ethanol to a concentration of not over than 0.1% (v/v). The drug was treated at 10 day after seeding cells. Verapamil concentration was chosen at

50, 100, 200, 500, and 1000 nM based on FDA approved IC₅₀ values [14]. The contraction force decreases relatively linearly with drug concentrations. When applying drug concentration up convenient drug dose, initial displacement has no significant changes during measurement cycle in both cases. The contraction force of the cardiac cell was reduced nearly 20% at 200 nM concentration without electrical stimulation (Fig. 5(a)). Also beating periods were not constant in the absence of electrical stimulation. In contrast, when applying electrical field to the contracting CMs, contraction force was decreased up to 50% at the same concentration of the drug (Fig. 5(b)) and the beating frequency was kept at 0.5 Hz during the measurement.

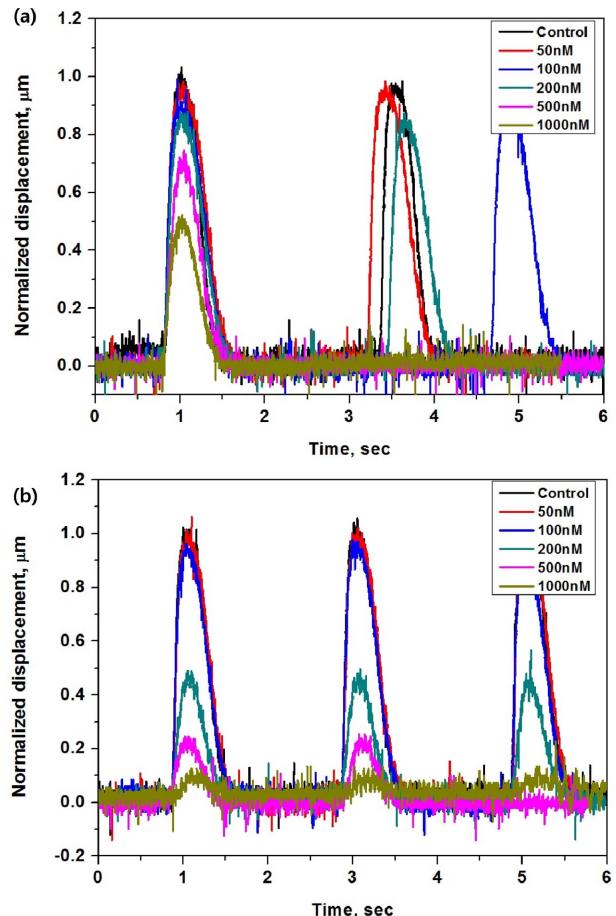


Figure 5: Normalized displacement changes due to the drug concentration (Verapamil -a calcium channel blocker). (a) Without electrical stimulation with drug concentration (b) With electrical stimulation with drug concentration

The cantilever showed a big difference in the vertical displacement depending on electrical stimulations. In order to verify this experimental result, we have performed immunocytochemistry (ICC) to detect the transmembrane expression of proteins, that is, heterohexameric arrays of connexins in the membrane of the closely neighbored cells [15]. The ICC result is shown in Fig. 6 which investigated the effect of the electrical stimulations on the expression of Cx-43. The electrical stimulation led to an increase in the number of dots (green) for Cx-43 in cardiac primary cell compared to cells without any electrical stimulation. Also

F-actin (red) was much more polarized and elongated under the electrical stimulation.

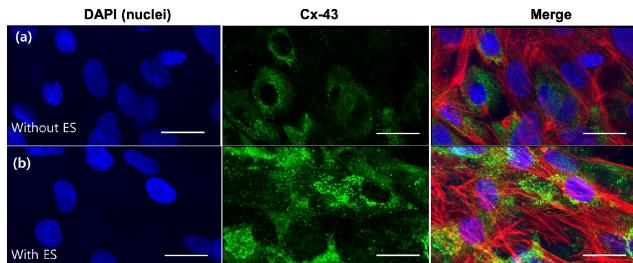


Figure 6: Fluorescence micrographs of cardiac primary cells. (a) in the absence of electrical stimulation and (b) in the presence of electrical stimulation. Nucleus (blue), Cx-43 gap junction protein (green) and F-actin (red), scale bar 20 μ m.

The morphological and functional alterations are induced by electrical stimulation to cardiac primary cells. The electrophysiological maturation is required for *in vitro* tissue engineering of CMs. Electrical coupling could be enhanced between two cell communications and produce larger displacements. Therefore, the electrical stimulation is a valuable method for drug toxicity screening with *in vitro* CMs.

CONCLUSION

In this study, we designed and characterized a biocompatible polymeric cantilever integrated with an electrical stimulator. Cantilever surfaces with patterned micro grooves could effectively align CMs and enhance their contraction forces up to 3 times as compared to those from the cardiac cells on the flat cantilever. The two parallel metal electrodes enhanced the expression of gap junction proteins that can represent the cell maturation. In the cardiac toxicity testing, the electrical stimulation allowed us to monitor the change in the displacement more consistently in a function of the drug concentration. Our advanced cantilever system with functions of electrical and mechanical stimulations has a great potential to monitor CMs cultured in the environment mimicking the *in vivo* condition or their responses to drugs triggering cardiac toxicity.

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