Sensors and Actuators A 301 (2020) 111760

Contents lists available at ScienceDirect

Sensors and Actuators A: Physical

journal homepage: www.elsevier.com/locate/sna

Drug-induced changes in mechanical behavior of electrically synchronized cardiomyocytes on surface-patterned polydimethylsiloxane diaphragm

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ARTICLE INFO

Article history:
Received 7 June 2019
Received in revised form 17 October 2019
Accepted 18 November 2019
Available online 29 November 2019

Keywords:
Stage-top bioreactor
Electrical stimulator
PDMS diaphragm
Maturation
Cardiomyocytes drug screening

ABSTRACT

We herein describe the fabrication and evaluation of an electrical stimulator-integrated functional polydimethylsiloxane (PDMS) diaphragm platform to examine the influence of drug toxicity on electrically synchronized cardiomyocytes. The integrated electrical stimulator allows the cardiomyocytes to contract and relax at a specific beat frequency. The growth conditions of the cardiomyocytes can be modulated by controlling the duty cycle, pulse duration, and frequency of the electrical stimulator. Electrical stimulation not only synchronized the beat cycle of the cardiomyocytes but also affected cardiomyocyte maturation. The microgroove structures formed on the PDMS surface had a positive influence on the contractile force through the alignment of the cardiomyocytes. The displacement of the PDMS diaphragm caused by the contractile characteristics of the cardiomyocytes was precisely measured at the nanoscale using a laser displacement sensor. After preliminary experiments were carried out using the platform, two typical cardiovascular drugs (Verapamil and E-4031) were selected to evaluate the performance of the proposed sensor platform. The changes in contraction and beat rate due to drug toxicity were systematically investigated in the electrically synchronized and spontaneously beating cardiomyocytes on the PDMS diaphragm. The use of electrically synchronized cardiomyocytes in drug toxicity screening may contribute to elucidating the relationship between cellular contraction and drugs.

1. Introduction

Drug-induced cardiac toxicity is an important cause of failure in drug development and clinical trials. The problem of drug toxicity has led to the withdrawal of existing commercial cardiovascular or non-cardiovascular drug products from the market [1]. To address this issue, regulatory agencies such as the Food and Drug Administration and the European Medicines Agency demand in-vitro testing of drug candidates to identify potential risks of QT interval extension before clinical trials. Patch-clamp technology has been adopted as a gold standard in drug toxicity screening to measure QT interval extension at the cellular level [2,3]. However, electrophysiological analysis based on the patch-clamp requires skilled researchers and is not suitable for drug testing at the initial stage of drug development, which requires high throughput for fast screening. To overcome the drawbacks of the patch-clamp, a new electrophysiological method based on electrode arrays known as the microelectrode array has been developed to map the extracellular action potential of cardiomyocytes. The microelectrode array system enables local detection of the field potential of individual cardiomyocytes with high resolution and sensitivity but is unable to directly measure their contractile force [4–6].

The most vital function of the heart is to circulate blood throughout the body. To do this, regular contraction and synchronization of cardiomyocytes are essential features of cardiac function and depend on the electromechanical dynamics of cardiac tissue. While the evaluation of changes in the beat rate and contractility of cardiomyocytes upon drug intervention is not a requirement in preclinical safety guidelines, some have argued that it is essential to evaluate cardiomyocyte contractility during the early stages

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https://doi.org/10.1016/j.sna.2019.111760
0924-4247/

of drug development and selection. This is because conventional electrophysiological methods are considered as the only method in cardiac toxicity screening. Various approaches to analyze the mechanical behavior of cardiomyocytes in association with drug concentrations have been studied. For example, the mechanical behavior of single cardiomyocytes can be measured microscopically by observing changes in the curvature of micropost arrays induced by cell contraction. Each post of the arrays flexes independently in response to contraction of the attached cardiomyocytes, and the contractile force is measured by the degree and direction of bending of each post [7,8]. However, it is difficult to analyze the mechanical behavior of cardiomyocytes in real-time using this procedure, as images are acquired by optical microscopy. Furthermore, the bending of the posts is minute, making it difficult to systematically analyze the micropost displacement.

An alternative technique that offers better sensitivity is to measure the deformation of a cantilever structure using a monolayer of cardiomyocytes. The displacement of the cantilever caused by the contraction of the cardiomyocytes can be characterized using optical imaging, a laser displacement sensor, or an integrated strain sensor [9–11]. Various studies have been carried out on the relationship between cardiomyocyte contractility and drug-using cantilever devices. Although good new results have been obtained, the use of cantilever devices still has drawbacks. Since the cantilever has a suspended structure, it is difficult to maintain constant cell density on the cantilever. This is due to the loss of cardiomyocytes to the lower space rather than the upper part of the cantilever during the cell seeding process. Another issue is that the beat frequency of the cardiomyocytes in the control state is not always constant. This may lead to unexpected problems during the analysis of drug toxicity based on contractile forces. Besides, it is difficult to systematically analyze the beat rate and spontaneous contractive force, which are decreased by the drug, especially at a low beat rate.

Here, electrical stimulator-integrated polydimethylsiloxane (PDMS) diaphragm was employed for cardiac toxicity screening applications. The proposed platform is capable of electrically stimulating cultured cardiomyocytes through an integrated Au electrode. Real-time force analysis can be realized by measuring the displacement of the thin PDMS diaphragm induced by cell contraction using a laser vibrometer. Moreover, microgrooves formed on the upper part of the PDMS diaphragm improved contractility by aligning the cardiomyocytes. For preliminary drug toxicity screening, further experiments were conducted using two drugs. The difference between spontaneous and synchronized beating was studied based on the changes in the beat frequency and contractile force of cardiomyocytes.

2. Design and fabrication

Fig. 1 shows a schematic of the electrical stimulator-integrated functional PDMS diaphragm and the principle of measuring contractility based on cell relaxation. The PDMS diaphragm, which possesses biocompatible and elastic properties, was employed to analyze the cell contractile force by measuring the diaphragm displacement. The beat frequency of the cultured cardiomyocytes was controlled by electrical stimulation, and the electrodes required for this function were formed on a glass substrate employed as a body. The cardiomyocytes were radially aligned along the micropatterns formed in the PDMS diaphragm, which maximized the displacement of the diaphragm as the cells contracted and relaxed. Four diaphragms were formed on a single chip to improve the accuracy of the analysis. The size of the proposed electrical stimulator-integrated PDMS diaphragm structure was 14 mm × 14 mm × 0.5 mm, with a thickness and diameter of 10 μm and 3 mm, respectively. The distance between the two electrodes for electrical stimulation was 12 mm. The microgrooves formed on the surface of the PDMS diaphragm were diagonally aligned to the center of the diaphragm in the quadrant. The grooves had a width of 3 μm, a depth of 0.5 μm, and a gap distance of 3 μm. To measure the vertical displacement of the diaphragm caused by cardiomyocyte contraction and relaxation using a laser displacement sensor, a circular reflector with a diameter of 500 μm was formed on the bottom surface of the diaphragm. Finite element analysis was performed using COMSOL Multiphysics to confirm the displacement change based on the force applied to the PDMS diaphragm where the cardiomyocytes were cultured. The PDMS film was deformed in the vertical direction by using a force in the normal direction (Young's modulus: 750 kPa, Poisson's ratio: 0.49). Simulation results showed that as the applied force increased from 10 nN to 100 nN, the displacement of the PDMS diaphragm increased from 1.3 μm to 13.1 μm. The calculated spring constant of the PDMS diaphragm was 7.63 μN/m (Fig. S1). The PDMS diaphragm displacement could be precisely measured at the nanoscale using a laser vibrometer, which exhibits exceptionally high sensitivity compared to that of integrated strain sensors.

Fig. 2 shows the fabrication process of the electrical stimulator-integrated functional PDMS diaphragm. The proposed PDMS diaphragm structure consisted of microgrooves for cell alignment, a PDMS layer integrated with a reflector for displacement measurement, and a glass body with two electrodes for electrical stimulation. During the fabrication process, a microgroove mold was formed by patterning photoresist on a Si wafer using a photolithographic process to obtain 3-μm lines and spaces. A thin PDMS layer was formed on the microgroove mold via spin-coating. The thickness of the PDMS diaphragm was 10 μm, and the spin-coated PDMS layer was cured at 80 °C for 1 h using a hot plate. After deposition of 10 nm of Ti and 100 nm of Au using an E-beam evaporator, a reflector was defined on the PDMS layer through photolithographic and metal etching processes [12]. The shape of the PDMS diaphragm was then defined by cutting the PDMS layer using a blade. Two electrodes on the glass part were also defined with 10 nm of Ti and 100 nm of Au using an E-beam evaporator, and the glass part with electrodes was through-etched to form holes with diameters of 3 mm. A sand-blasting process was employed for glass etching. Next, a glass body (14 mm × 14 mm) with electrodes and holes was diced from a glass wafer. The Si wafer with the functional PDMS diaphragm and glass body with two electrodes were bonded using a plasma surface treatment system (FEMTO SCIENCE, CUTE-MPR). The fabrication of the electrical stimulator-integrated functional PDMS diaphragm was completed by removing the photoresist used as the microgroove mold.

3. Results and discussion

3.1. Effect of microgrooves on cardiomyocyte growth

Microstructures formed on the surface of the diaphragm have a significant influence on sarcomere length, which is an essential factor affecting the contractile force of cardiomyocytes. In this experiment, microgrooves with a width and spacing of 3 μm were integrated to maximize the displacement of the PDMS diaphragm. Fig. 3 shows optical images of cardiomyocytes cultured on the PDMS diaphragms with and without microgrooves and the displacement of the PDMS diaphragm through cardiomyocyte contraction and relaxation on day 9. The experimental results showed that cardiomyocytes cultured on the PDMS diaphragm without microgrooves were grown isotropically. The displacement of the PDMS diaphragm caused by cardiomyocyte contraction and relaxation was 4 μm. On the other hand, cardiomyocytes cultured

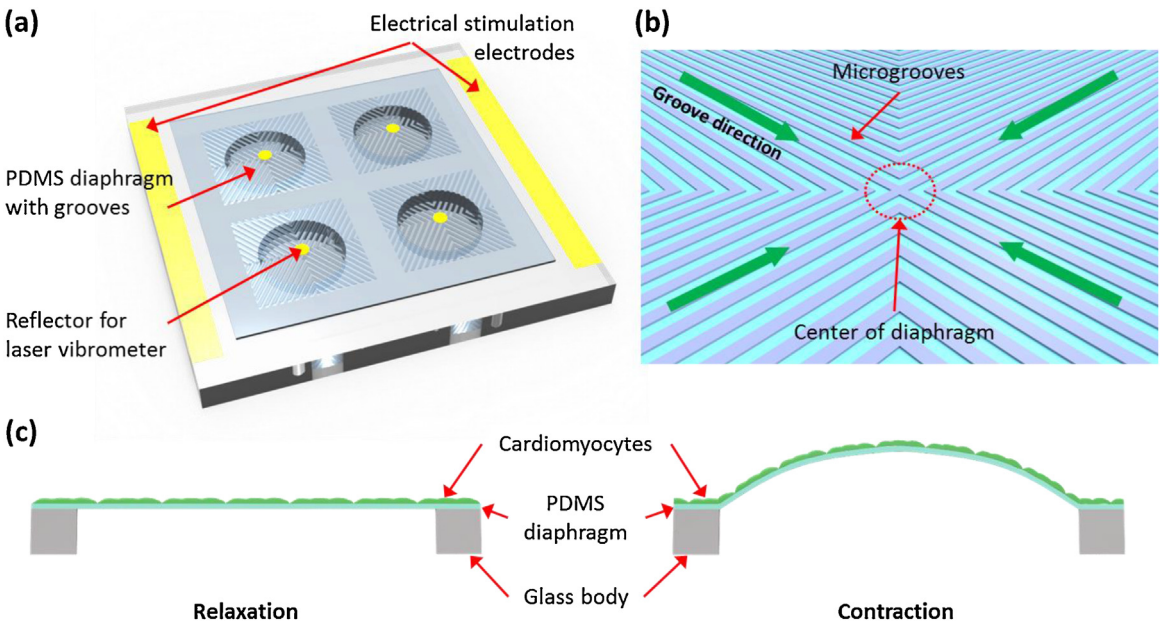

Fig. 1. Schematic diagram of the electrical stimulator-integrated functional PDMS diaphragm.

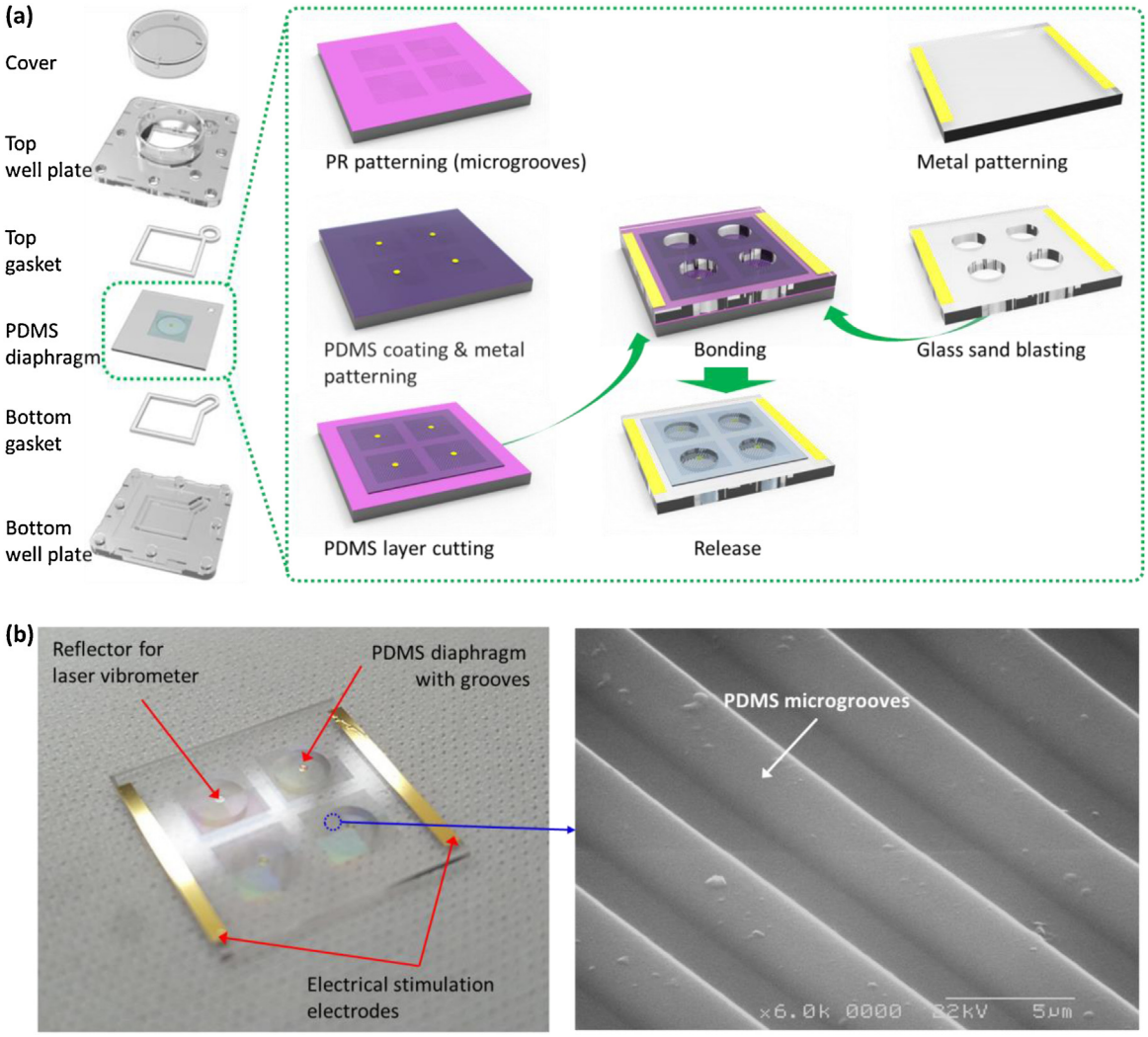

Fig. 2. (a) Fabrication process of the electrical stimulator-integrated functional PDMS diaphragm composed of silicon and glass parts. (b) Optical image of the fabricated functional diaphragm and scanning electron microscopic image of the PDMS diaphragm surface with microgrooves.

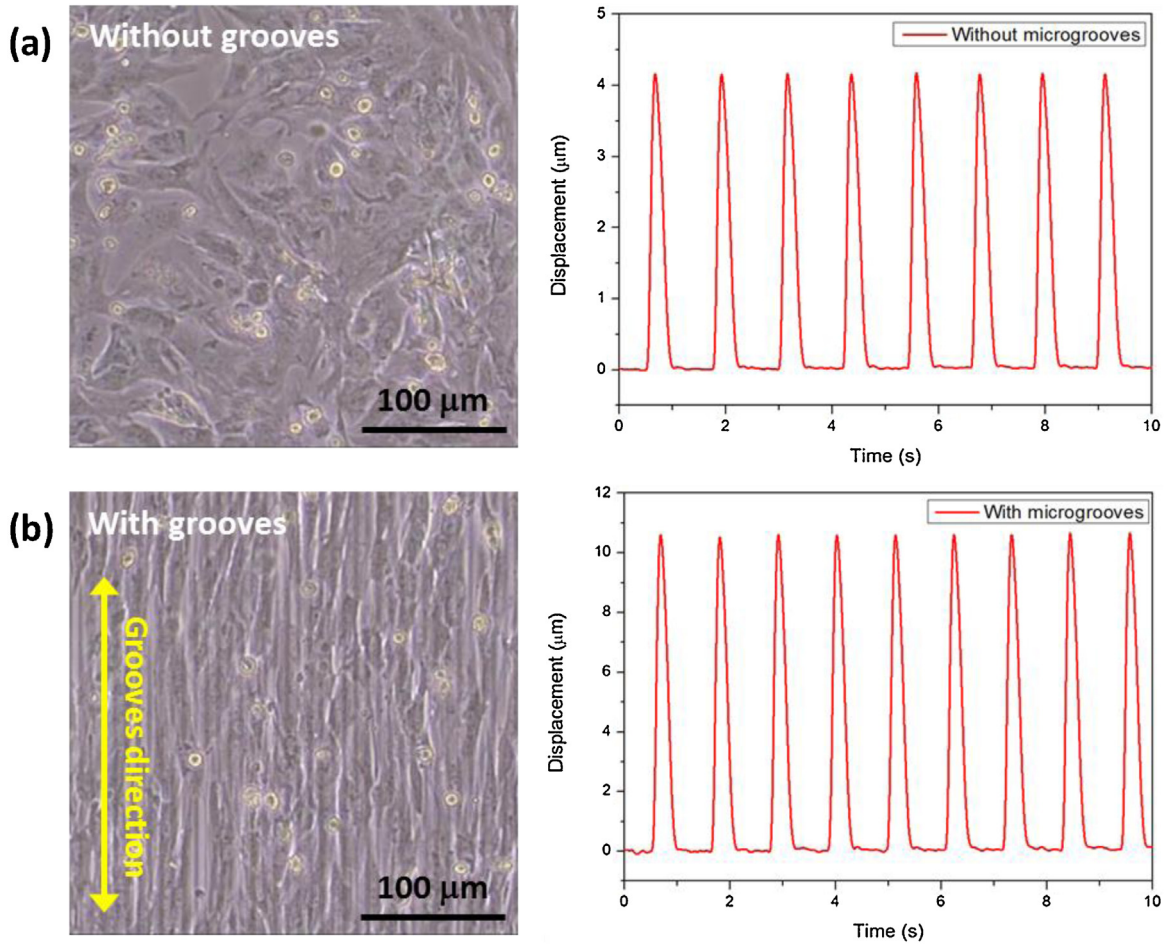

Fig. 3. Optical images and contraction behavior of cardiomyocytes on PDMS diaphragms (a) without and (b) with microgrooves.

on the PDMS diaphragm with microgrooves were well aligned in the groove direction, and the displacement was measured to be 10.6 μm. As the microgrooves were formed in the radial direction of the diaphragm, the cardiomyocytes showed better alignment in the radial direction of the PDMS diaphragm (Fig. S2), and the enhanced contraction behaviors caused the displacement of the PDMS diaphragm to increase by 2.6 times (Fig. 3).

Contractility of the cardiomyocytes cultured on the microgroove-patterned PDMS diaphragm was investigated by measuring the displacement of the PDMS diaphragm. Fig. S3a in the supporting information shows the displacement of the cardiomyocytes seeded microgroove-patterned PDMS diaphragm. The ideal relative contraction force and a beat rate of the cultured cardiomyocytes and ideal displacement of the cardiomyocytes cultured microgroove-patterned PDMS diaphragm were measured by using five functional well-plates and all the obtained data are expressed as mean ± s.d. for at least five independent experiments. The displacement of the microgroove-patterned PDMS diaphragm gradually increased with increasing the culture period. The microgroove-patterned PDMS diaphragm showed the maximum displacement of 9.485 ± 1.485 μm on day 9 of the culture period. The relative contraction force of the cultured cardiomyocytes on day 9 was found to be 477.8 ± 74.8 (Fig. S3b). Then, the displacement of the PDMS diaphragm and relative contraction force of the cardiomyocytes decreased with further increasing the culture period. This phenomenon could be attributed to the finite lifetime of the cultured cardiomyocytes. Besides the beating rate of the cultured cardiomyocytes decreased at higher culture period indicative of more maturation of cardiomyocytes (Fig. S3c) resembling studies based on NRVM tissue [13]. The beat rate of the cardiomyocytes on day 3 was found to be 129 ± 9, and it was decreased ~60 % (25 ± 7) on day 9. We have carried out the Immunocytochemistry (ICC) staining analysis to demonstrate the improved maturation of the cardiomyocytes at higher culture period. Fig. S3d shows the α-actinin of cardiomyocytes and sarcomere length of the cardiomyocytes on day 5 and 9 of the culture day. The α-actinin of the cardiomyocytes attained from day 9 more elongated in the microgroove direction compared to that of cardiomyocytes obtained from day 5. The sarcomere length of cultured cardiac cells on day 5 and day 9 was found to be ~ 1.79 ± 0.0047 μm and 1.88 ± 0.0045 μm. The higher sarcomere length of cardiomyocytes on day 9 was indicating the formation of more matured cardiomyocytes resembling the previous investigation [14–16]. Besides, the displacement of the four diaphragms formed on a single chip, caused by cell contraction, on the 9th day of cell culture showed a difference of ±3.7 %, which was expected to be caused by the difference in cell density (Fig. S4). The proposed diaphragm structure shows a minute difference compared to the conventional cantilever sensors.

3.2. Synchronization of cardiomyocyte beating by electric stimulation

The electrical stimulator-integrated functional PDMS diaphragm was used to synchronize the beating of cardiomyocytes through electrical stimulation and to measure the diaphragm displacement caused by cell contraction and relaxation. Fig. 4a shows the schematic illustration of the experimental setup used for mea-

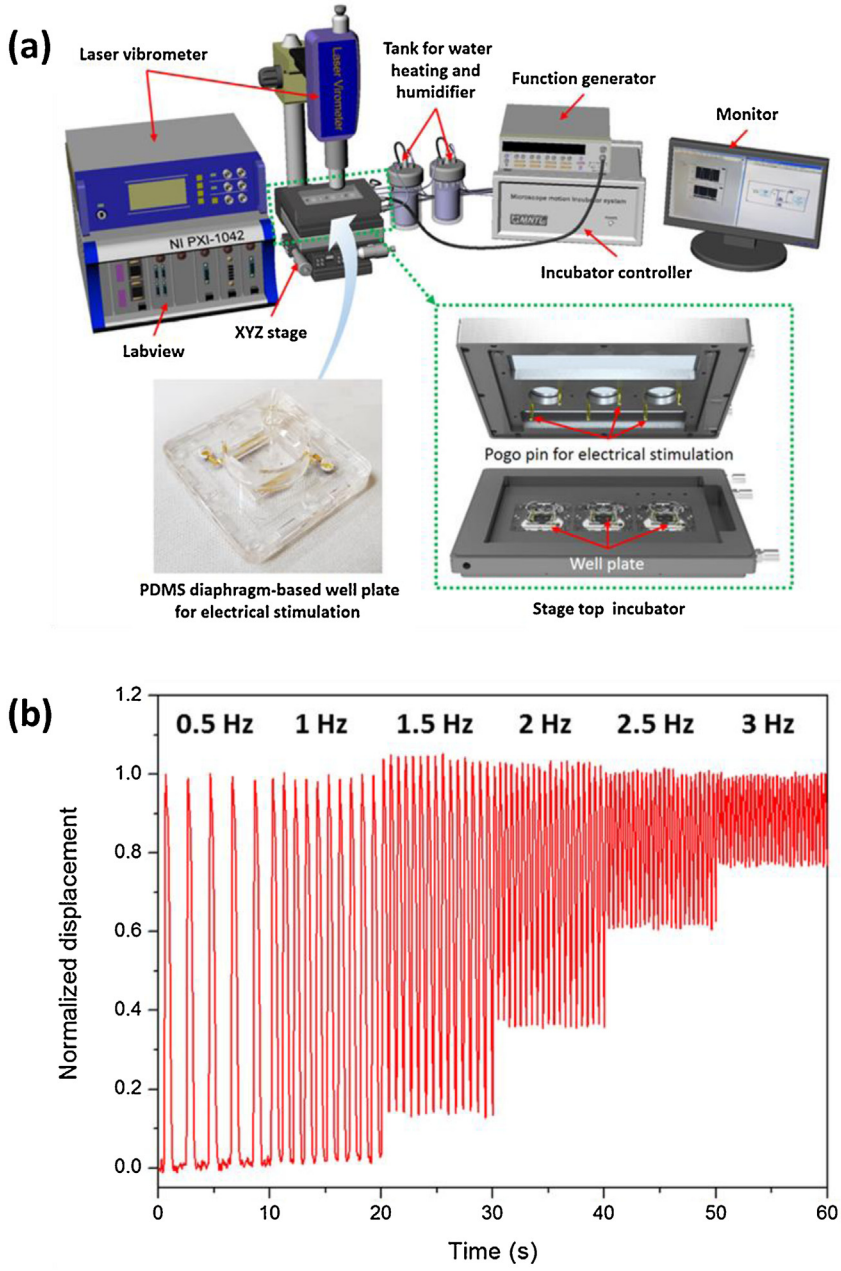

Fig. 4. (a) Schematic illustration of the measurement system used for electrical stimulation. (b) Contraction and relaxation analysis of cardiomyocytes at various electrical stimulation frequency.

suring the contraction force of the cardiomyocytes cultured on the proposed PDMS diaphragm. The measurement system consisted of a function generator for electrical stimulation, a charge-coupled device-laser displacement sensor (LK-G30, Kenyence, Osaka, Japan) controlled by Lab-VIEW for measuring the displacement of the PDMS diaphragm, and a home-made stage-top incubator. The X-Y-Z stage was used to align the laser spot of the displacement sensor to the metal reflector integrated on the PDMS diaphragm. Electric stimulation to synchronize cell beating was performed using square-wave pulses at a pulse duration of 2 ms and an electric field of 3 V/cm. Preliminary analysis of the maximum force and single beat shape of cardiomyocytes showed no significant changes with and without electrical stimulation, as shown in S5.

Fig. 4b shows the normalized displacement of the cardiomyocytes cultured PDMS diaphragm as a function of different external electrical stimulation. The beat rate and contraction characteristics of the cardiomyocytes in response to the change in electrical stimulation frequency (0.5–3 Hz) were further analyzed. The beat rate was precisely synchronized to 30, 60, 90, 120, 150, and 180 beats/min as the electric stimulation frequency increased from 0.5 Hz to 3 Hz. It was also confirmed that the cells were not completely relaxed and contracted when the beat frequency was higher than 1 Hz. In other words, as the beat frequency increased, the maximum displacement of the PDMS diaphragm was kept constant at 10 μm, while the change in displacement decreased. Thus, it was difficult to accurately analyze the diaphragm displacement induced by cardiomyocyte contraction and relaxation when the electric stimulation frequency was 1 Hz or higher. Also, the contractile force of the cardiomyocytes was the highest 9–10 days after cell culture, and the beat frequency at this time was 0.4 Hz. Therefore, cardiomyocyte synchronization through electrical stimulation was carried out at a frequency of 0.5 Hz.

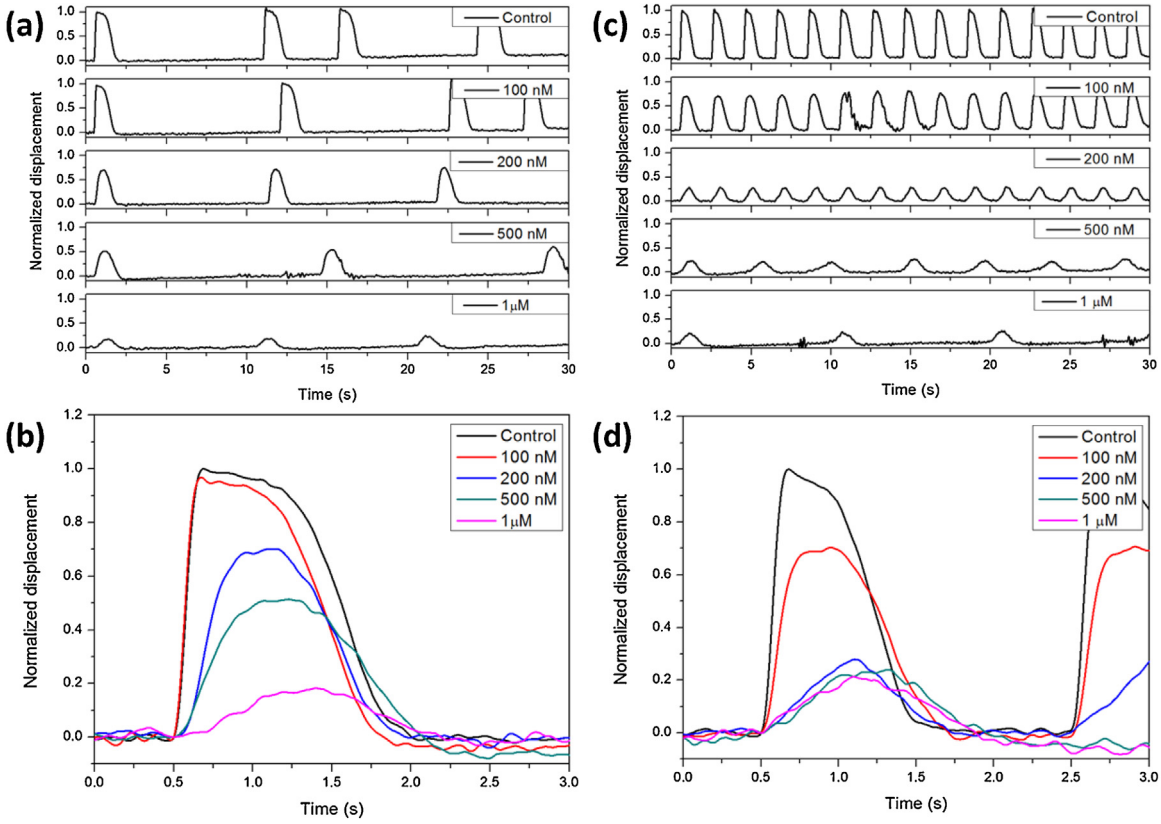

Fig. 5. Changes in beat frequency and contractile force of cardiomyocytes with and without electrical stimulation as a function of Verapamil concentration. (a, b) without stimulation, (c, d) with stimulation.

3.3. Dose-response studies of cardiac drugs

Cardiomyocytes were obtained from neonatal rat ventricular myocytes, as described in previous studies with the approval of the animal ethics committee of Chonnam National University [17–21]. Drug toxicity experiments were performed with Verapamil (Sigma-Aldrich), an L-type Ca^{2+} channel blocker, and E-4031 (Sigma-Aldrich), a human ether-a-go-go-related gene (hERG) channel blocker. The contractile force and beat frequency of the cardiomyocytes were measured after 9 days of cell culture with drugs at various concentrations. The ethanol in which the drug was diluted was maintained at a concentration below 0.1 % (v/v). Drug concentrations were selected based on IC_{50} values approved by the Food and Drug Administration.

The changes in beat frequency and contractile force of the cardiomyocytes with changes in drug concentration were analyzed with and without electrical stimulation. Generally, Verapamil is used to control the ventricular rate in the prevention of supraventricular tachycardia, as the contraction and beat rate decrease as the drug concentration increases [22]. Fig. 5a and c show the changes in cardiomyocyte beat rate with the administration of various concentrations of Verapamil with and without electrical stimulation. Spontaneously beating cardiomyocytes showed slightly reduced beat rate with increasing concentrations of Verapamil, while electrically stimulated cardiomyocytes maintained a beat rate of 30 beats/min up to a concentration of 200 nM. At 500 nM, the beat rate was not synchronized by electrical stimulation and decreased to 14 beats/min (Fig. S1a). The beat rate of electrically stimulated cardiomyocytes at 1 μM was 6 beats/min, which was similar to that of spontaneously beating cardiomyocytes. As shown in Fig. 5b and d, as the concentration of Verapamil increased, the displacement of the PDMS diaphragm associated with cardiomyocyte contractility was equally reduced in spontaneously beating and electrically stimulated cardiomyocytes. Compared with cardiomyocytes with a synchronized beating at Verapamil concentrations of 100 nM, 200 nM, and 500 nM, spontaneously beating cardiomyocytes showed significantly decreased contractile force. When the concentration of Verapamil was 1 μM, both types of cardiomyocytes showed similar contraction characteristics, which were almost the same as those in the necrotic state as they were decreased by more than 80 %.

E-4031 is a class III antiarrhythmic drug that blocks hERG-type potassium channels and prolongs repolarization. It is used for research purposes only because it can cause fatal arrhythmia by prolonging the QT interval. Fig. 6a and c show the changes in beat rate after cardiomyocytes were subjected to various concentrations of E-4031 and washout periods, both with and without electrical stimulation. The beat rate of spontaneously beating cardiomyocytes gradually decreased with increasing concentrations of E-4031, to approximately 44 % at the maximum concentration of 50 nM and slightly increased after the culture medium was replaced. Electrically stimulated cardiomyocytes exhibited constant beating in synchronicity when the E-4031 concentration was 5 nM and 10 nM. However, at concentrations above 20 nM, there were intermittent abnormal beats with synchronized beats by electrical stimulation (Fig. S6b). One day after the culture medium was replaced, intermittent beats that were no longer synchronized with electrical stimulation were observed. Two days after medium change, the beats were synchronized by electrical stimulation, but abnormal contraction characteristics were observed at the maximum contraction point of every single beating, similar to samples that were not electrically stimulated. The contractile force of cardiomyocytes with and without electrical stimulation did not show gradual changes, as shown in Fig. 6b and d, but changed within

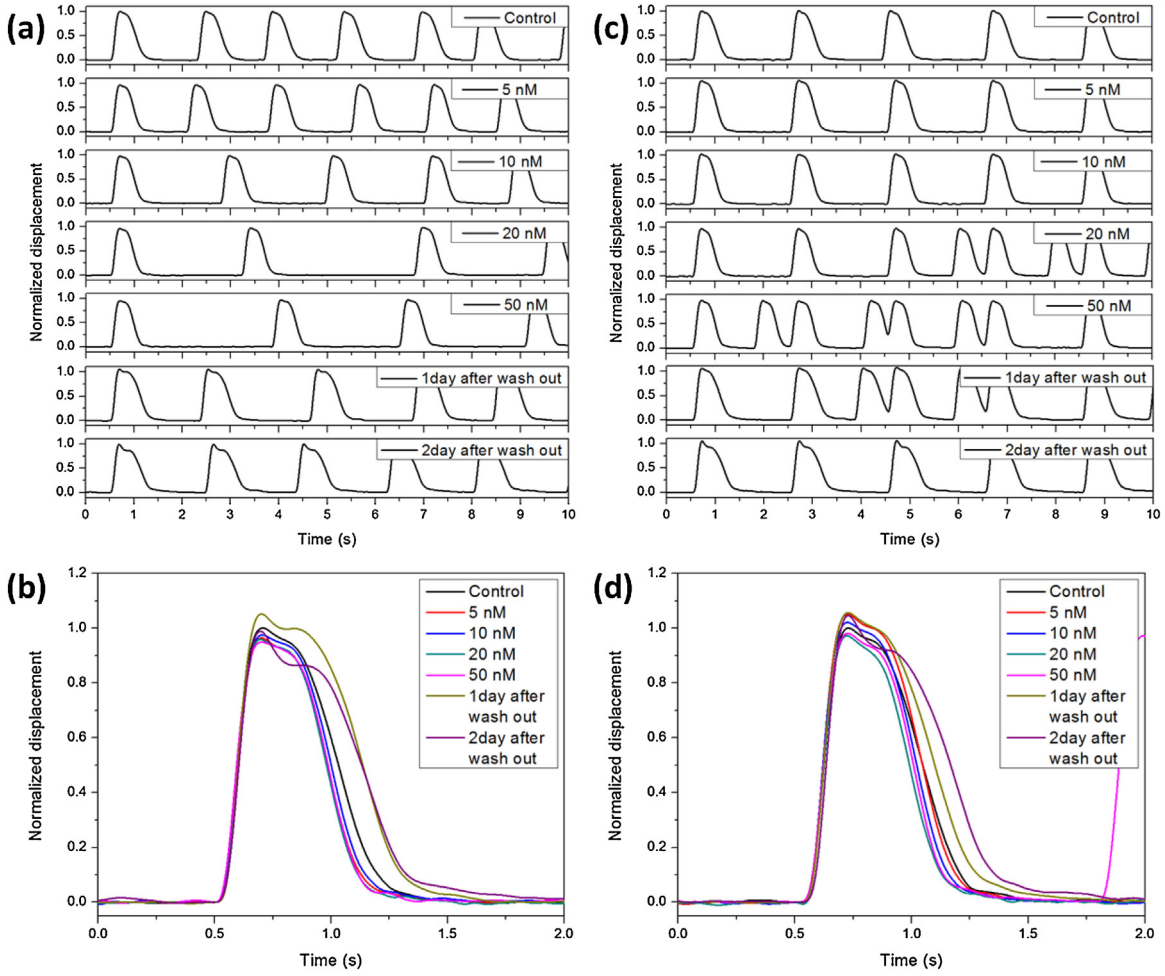

Fig. 6. Changes in beat frequency and contractile force of cardiomyocytes with and without electrical stimulation as a function of E-4031 concentration. (a, b) without stimulation, (c, d) with stimulation.

5 % with increasing drug concentration. From these experimental results, it can be seen that E-4031 is not related to calcium ions, which has a large influence on contraction.

The preliminary experimental results showed that the electrically stimulated cardiomyocytes exhibited reduced contraction with increasing concentration of Verapamil, a Ca^{2+} channel blocker. The contraction was not synchronized with electrical stimulation at a concentration close to the IC_{50} measured by conventional electrophysiology [23]. In contrast, E-4031, an hERG channel blocker, exerted no effect on the contractile force with increasing concentration. Aside from that induced by electrical stimulation, E-4031 caused an additional beating at a concentration similar to the IC_{50} value measured by electrophysiology, resulting in an increase in beat rate. Cardiomyocyte contraction is initiated directly as Ca^{2+} is introduced through L-type Ca^{2+} channels in the cell membrane, thereby increasing the concentration of Ca^{2+}. In the presence of Ca^{2+} channel blockers, the concentration of Ca^{2+} in the cardiomyocytes is decreased. It is expected that the contractile force would also decrease and that stimulation would not induce beating. Generally, most cardiomyocytes isolated from animals exhibit irregular beating characteristics during incubation. Thus, it is challenging to resolve the issue of drug toxicity based on contraction behaviors and beat cycles. The preliminary results obtained using the proposed method involving electrical stimulation exhibited great potential in addressing the drawbacks of previous methods since it is possible to control the beat cycle of cardiomyocytes at a constant level.

4. Conclusion

In this study, a functional PDMS diaphragm integrated with an electric stimulator was employed to synchronize the beat rate of cardiomyocytes through electrical stimulation, and drug-induced cardiac toxicity of the electrically synchronized cardiomyocytes was analyzed in real-time. The electrical stimulation conditions were optimized at a frequency of 0.5 Hz, pulse duration of 2 ms, and electrical field of 3 V/cm. The contraction and synchronization characteristics of drug-induced cardiomyocytes with and without electrical stimulation were systematically investigated using the proposed drug toxicity screening platform. According to the primary experimental results, the beat synchronization of cardiomyocytes by electrical stimulation can solve the irregular beat issue of previous drug screening studies based on contractile force. Besides, the exciting results were that the cardiomyocytes exposed to a drug concentration similar to the IC_{50} value assessed by electrophysiological assays were not to synchronize the beat rate regardless of electrical stimulation. In the future, the proposed electrical stimulator-integrated functional PDMS diaphragm is expected to be useful in better understanding the electromechanical biological behavior of cardiomyocytes.

Declaration of Competing Interest

The authors declare that they have no conflict interests.

Acknowledgements

This study was supported by a National Research Foundation of Korea grant funded by the Ministry of Science and ICT (2017R1E1A1A01074550) and the Korea Health Technology R&D Project funded by the Ministry of Health & Welfare (HI19C0642), Republic of Korea.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.sna.2019.111760.

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