



Nano-textured polyimide cantilever for enhancing the contractile behavior of cardiomyocytes and its application to cardiac toxicity screening

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ABSTRACT

Polydimethylsiloxane (PDMS) cantilevers with hydrophobic nature have been extensively utilized in biosensors for their relatively good mechanical properties and biocompatibility. However, when nanostructures are formed on the surface of PDMS, it is difficult to observe the long-term behavior of cardiomyocytes because of the inherent hydrophobicity of the material. In this paper, we propose the use of nanotextured polyimide (PI) cantilevers to measure cardiac contractility. The proposed PI material not only has very high biocompatibility, but also has high thermal resistance which is useful for metal deposition and sterilization processes. Additionally, an oxygen-plasma-treated PI cantilever surface provides more suitable environment for cardiomyocytes that have a sarcomere length greater than 2.0 μm , which is close to the size of mature adult cardiomyocytes. Immunofluorescence experiments were conducted to analyze the biological characteristics of cardiomyocytes grown on the nanotextured PI cantilevers. Following these preliminary experiments, the fabricated PI cantilevers were utilized to measure the contractile behavior of drug-treated cardiomyocytes. The different contractile behaviors of cardiomyocytes grown on PDMS and PI cantilevers were also compared using a laser-based displacement sensor. We believe that the nanotextured PI cantilever has significant potential for basic studies on the contraction of matured cardiomyocytes and their interaction with drugs.

1. Introduction

Millions of people suffer from cardiovascular diseases for a variety of different reasons, including the side effects of drugs [1,2]. In the pharmaceutical industry, the patch clamp is a widely used and reliable electrophysiological method of drug toxicity assessment. A patch clamp operates based on the working principle of changes in the action potentials of individual cardiomyocytes [3]. Despite of the great success of the patch-clamp method in the field of drug-induced cardiac toxicity, this electrophysiological method still suffers from the need of highly skilled researchers, extremely low throughput and high cost etc. [4,5]. Furthermore, even some drugs which were successfully commercialized with the approval of the Food and Drug Administration have been canceled by the cardiac toxicity issues in practical use [6]. As this issue becomes more serious, various opinions and research results are being presented on the cause of the problem. One possible reason for this issue is the use of animal cells during the evaluation of cardiac toxicity. Another possible consideration is that the patch-clamp is the only

method used to determine the IC_{50} values of individual cardiomyocytes in the early stages of drug development [7,8].

Recently, several studies have attempted to evaluate the contractile force of cardiomyocytes quantitatively using microposts or microcantilever structures. The micropost arrays fabricated from elastomers measure the contractility of individual cardiomyocytes through the calculation of their structural deformation. However, the low resolution of optical images used for force analysis makes this method relatively unreliable [9,10]. The microcantilever structures can measure the contractile force of cardiomyocytes more accurately [11–14]. This is because the cantilever surface employs a monolayer of cardiomyocytes. Recently, several studies have employed to evaluate the contractility of matured cardiomyocytes on the surfaces of cantilevers as a part of the drug toxicity screening process [15–17]. The cantilever technology has a significant advantage in that it can be used as a high-efficiency drug toxicity screening platform because it can simultaneously analyze various drugs or different concentrations of one drug by utilizing multiple cantilevers in parallel [18]. New analysis methods based on the

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contractility of cardiomyocytes are expected to provide useful information to complement the existing patch-clamp technique for evaluating drug toxicity.

Various polymer materials, such as hydrogel [19], cellulose [20], and polydimethylsiloxane (PDMS) [21], have been used in cardiomyocyte research. Specifically, PDMS has been widely used to measure the contractility of cardiomyocytes because it is easy to produce in the form of cantilevers and provides a low Young's modulus [22]. However, PDMS has low heat resistance, leading to surface cracking and deformation during the cantilever fabrication process. This feature makes it difficult to add functionality such as a strain sensor onto the cantilever surface [23,24]. Additionally, cell growth is nearly impossible without additional surface treatment due to the hydrophobic nature of the material [25]. Although extracellular matrix coatings are applied on the surface of PDMS, they often lead to serious issues, such as cell aggregation and detachment, when cardiomyocytes grow on PDMS surfaces for long time periods [26]. It also seems that the enhanced contractile force of grown cardiomyocytes tends to weaken the adhesion force between cells and PDMS cantilever surface. The hydrophobic nature of the PDMS substrate can be reduced by oxygen plasma treatments that activate functional polar silanol groups (SiOH) [27]. However, it easily recovers its hydrophobic status either over time or through heat treatment based on relocation of polar groups on the substrate surface [28]. The presence of functional groups ($\text{CH}_3 \sim \text{NH}_2 < \text{COOH} \sim \text{OH}$) on the substrate surface affects the adsorption and binding force of the fibronectin and integrin of the cell surfaces [29–31]. The hydrophobic surface of PDMS contains CH_3 functional groups, which are unsuitable for protein binding and cell attachment [32]. Polyimide (PI) is a polymer material that is also widely used in industry for construction, medical implants, and materials for optoelectronics. It has shown a biocompatible nature and good heat resistance up to 400 °C [33]. Additionally, oxygen plasma treatment of a PI substrate generates COOH or OH functional groups on the surface, which are helpful for cardiomyocyte attachment. Unlike conventional PDMS material, it maintains hydrophilic surface properties for more than one month [34,35].

In this paper, we report the fabrication of nanotextured PI cantilevers and their application to preliminary screening for cardiac toxicity. To analyze the characteristics of the PI material, cardiomyocytes grown on the nanotextured PI were analyzed using an optical microscope and immunohistochemistry. The contact angles of oxygen-plasma-treated PI cantilevers with nanotextures were characterized as a function of time. The experimental results are compared to those of PDMS cantilevers with and without nanotextures. In the case of the PI cantilevers, cardiomyocyte attachment is much stronger and growth is more active. Additionally, Verapamil-induced toxicity evaluation of cultured cardiomyocytes on the PI cantilever was performed using a laser vibrometer system. Changes in the contractile forces of cardiomyocytes with drug concentration were found to exist in various forms, such as single-bit shapes, beating cycles, and cantilever displacement. Furthermore, a strain sensor can be integrated onto the PI cantilever, enabling parallel operation for high-throughput screening applications.

2. Materials & methods

2.1. Design and fabrication of nanotextured PI cantilevers

Fig. 1 presents a schematic of a drug-induced cardiac toxicity screening platform based on nanotextured PI cantilevers and a laser vibrometer system (OFV-534, Polytec). Cardiomyocytes were obtained from a neonatal rat ventricular myocyte (NRVM) isolation protocol and immediately cultured on the PI cantilevers. The nanotextured PI cantilevers with cardiomyocytes were placed in a stage-top incubator (Chamlide WP, Live Cell Instrument) with a temperature of 37 °C, CO_2 concentration of 5%, and relative humidity of 100%. The nanotextures formed on the PI cantilevers significantly enhance the alignment of

cardiomyocytes, which also increases maturation and contractility. The cantilever displacement caused by relaxation and contraction of cardiomyocytes was recorded using the LabVIEW (Lab-VIEW 2009, National Instruments) program in vitro. Various concentrations of Verapamil (L-type calcium channel blocker, Sigma-Aldrich) altered the contraction properties of matured cardiomyocytes on the PI cantilever. The corresponding changes in cantilever displacement were systematically analyzed to evaluate the levels of cardiac toxicity.

Fig. 2 presents the process for the fabrication of the nanotextured PI cantilevers. First, a 4- μm -thick PI (DCFPI-301, Dongbaek Fine-Chem) layer is spin-coated onto a 12- μm -thick PI substrate (Kapton, SKC KOLON PI) (Fig. 2-1,2). Next, nanogrooves with widths of 800 nm are patterned on the PI layer using a PDMS stamp with nanotextures and a baking process at 200 °C (Figs. 2-4 and Fig. S1). A metal reflector (Ti/Au 10 nm/90 nm) is then deposited on the nanotextured PI substrate using the electron-beam evaporation (Figs. 2-5). Next, the nanotextured PI substrate is cut by a Pinnacle die (BFX, TSUKATANI) with a sharp blade to form a pair of cantilevers (Figs. 2-6 and Fig. S2). Finally, a cantilever body is firmly fixed to a rigid support consisting of a glass plate (Figs. 2-7). Optical images of fabricated PI-cantilevers with dimensions of 6 mm \times 2 mm \times 16 μm (L \times W \times H) and bodies with dimensions of 10 mm \times 7 mm \times 3 mm are presented in Fig. 3. To analyze the physical properties of the fabricated PI-cantilevers, a resonance frequency measurement experiment based on the LabVIEW program was performed as shown in Fig. S3 (a). The Young's modulus and spring constant calculated from the resonance frequency were approximately 5 GPa and 47 mN/m, respectively, as shown in Fig S3 (b).

2.2. Cultivation of cardiomyocytes and cantilever displacement measurements

All experiments were conducted with approval from the Chonnam National University Animal Ethics Committee. Prior to the cultivation of cardiomyocytes, the PI cantilever surfaces were subjected to oxygen plasma treatment (CUTE-MPR, FEMTO SCIENCE) at 80 W for 30 s. The ventricle cardiomyocytes of three-day-old neonatal rats (Sprague Dawley Rat) were cultivated on the hydrophilic surfaces of the PI cantilevers based on NRVM isolation protocols [8,14,17,18]. PI cantilevers with cardiomyocyte seeding densities of 1000 cells/ mm^2 were placed in a 5% CO_2 incubator maintained at 37 °C. The culture medium for the cardiomyocytes was replaced every three days. The medium consisted of 67% Dulbecco's modified Eagle medium (DMEM, LONZA), 17% Heparin sodium salt from porcine intestinal mucosa (M199, Sigma-Aldrich), 10% Horse serum (HS, Sigma-Aldrich), 5% fetal bovine serum (FBS, Sigma-Aldrich), and 1% penicillin-streptomycin (P/S, Sigma-Aldrich). With the aid of metal reflectors located at the free ends of the PI cantilevers, the contractile properties of mature cardiomyocytes were recorded with nanoscale accuracy using a laser vibrometer system.

2.3. Immunocytochemical staining

On the seventh day of cultivation, the cardiomyocytes were fixed using a 3.7% formalin solution (Sigma-Aldrich) and washed three times with phosphate-buffered saline (PBS, Takara) for 10 min. They were then treated with 0.2% triton-X (Sigma-Aldrich) diluted in PBS for 15 min. The cell membranes were then blocked with 1% bovine serum albumin (1% BSA, Sigma-Aldrich) in a shaking incubator (VS-8480SF, Vision Scientific Co., Ltd.) at room temperature for 40 min. The primary antibody (α -sarcomere actinin, connexin-43) was then diluted at a ratio of 1:200 and treated at room temperature for 90 min. The secondary antibody (Alexa-Fluor 488 goat anti-mouse IgG conjugate and Alexa-Fluor 568 rabbit (1:200, abcam)) was diluted at a ratio of 1:500 and treated at room temperature for 60 min. Finally, staining was performed for 15 min at room temperature using 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and the samples were photographed

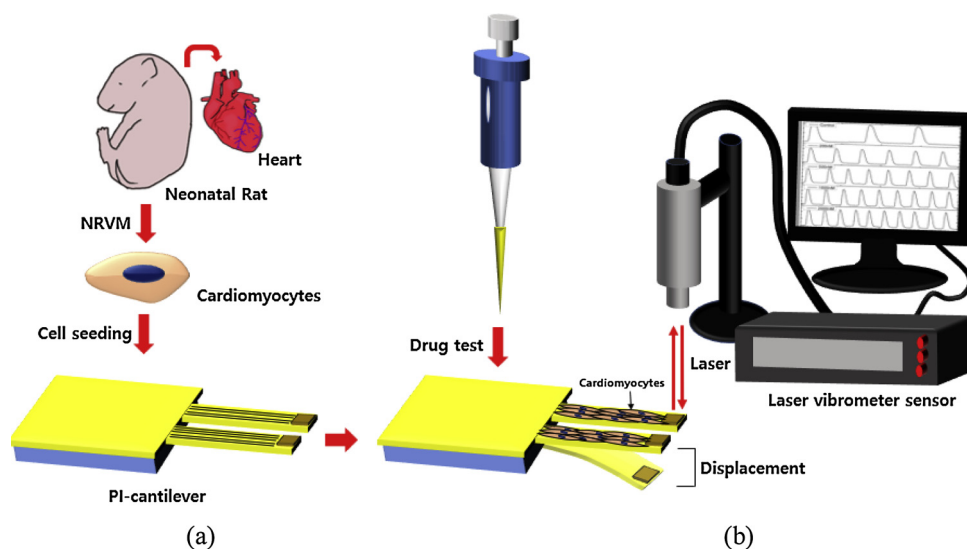


Fig. 1. Schematic of (a) a nanotextured PI cantilever for measuring the contractile force of cardiomyocytes and (b) its application to drug-induced cardiac toxicity screening.

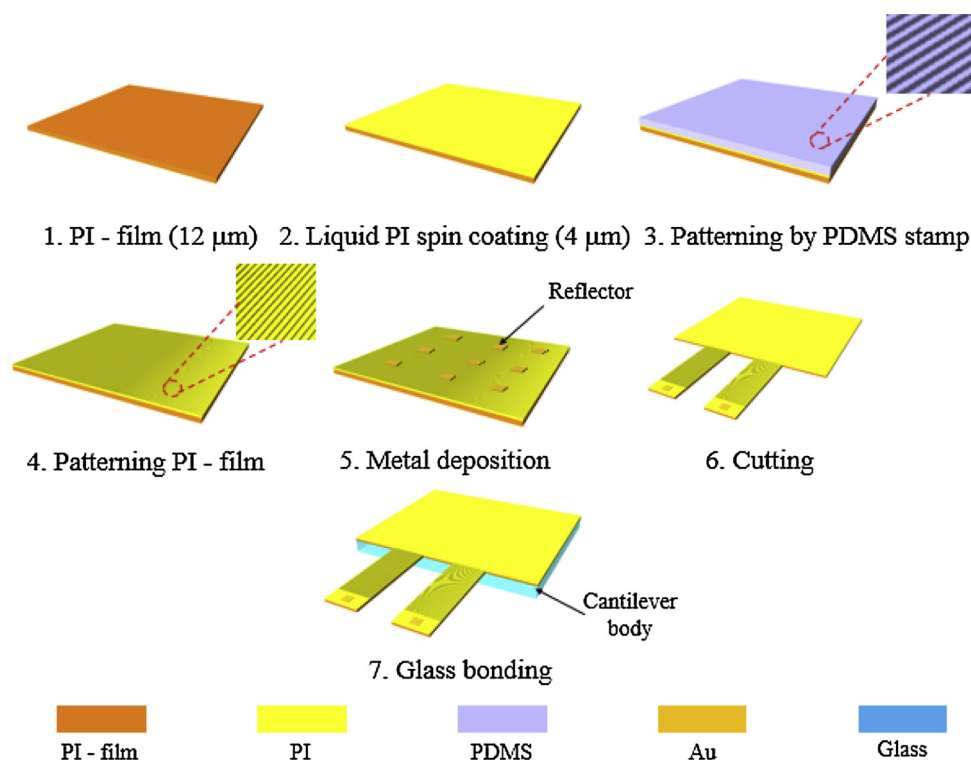


Fig. 2. Process flow for the fabrication of nanotextured PI cantilevers with a glass body. Two types of PI (solid and liquid), glass substrate and Au layer were employed in the PI cantilever fabrication.

using a confocal microscope (TCS SP5, Leica Microsystems GmbH) with an excitation wavelength of 488 nm and emission wavelength of 568 nm.

2.4. Water contact angle

The surface tension of the liquid phase determines the shape of a liquid droplet on a cantilever. Therefore, sessile drops were used to analyze the wettability of both of PI and PDMS nanotextured cantilevers for seven consecutive days. The volume of the water droplet was set to 5 μl to prevent it from spreading on the cantilever surfaces based on gravity. Each time a water droplet was placed on a cantilever

surface, the contact angle was measured after 20 s. The same experiment was repeated three times to ensure the reliability of the experimental results at a room temperature of 25 °C. These wettability tests for the PDMS and PI cantilevers were conducted based on contact angle measurements. Measurements were also performed before and after oxygen plasma treatment. Figure S4 presents the different contact angles of PDMS, silicone rubber, and PI cantilevers with flat and nanotextured surfaces. We confirmed that the contact angle increases significantly when nanotextures are fabricated on a surface. The experimental results for the contact angles of the flat PDMS cantilever, flat PI cantilever, nanotextured PDMS cantilever, and nanotextured PI cantilever are $114.4^\circ \pm 1.23^\circ$, $76.94^\circ \pm 4.48^\circ$, $126.2^\circ \pm 1.97^\circ$, and

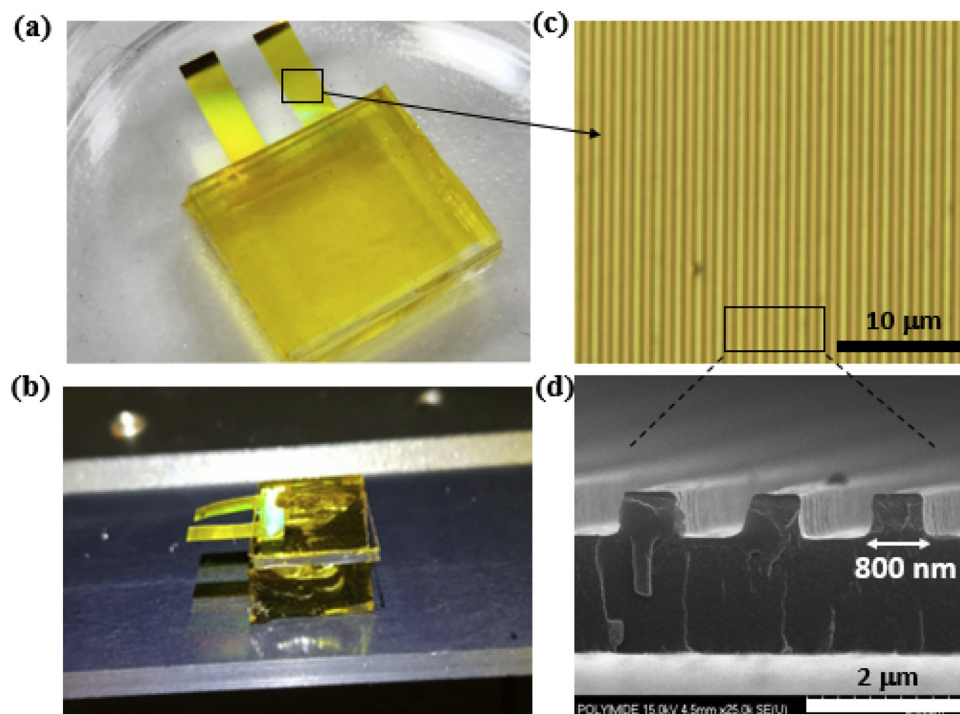


Fig. 3. Optical images of fabricated PI cantilevers with nanotextures: (a) top view, (b) side view, (c) magnified image of nanogrooves, and (d) scanning electron microscope image showing 800-nm groove width.

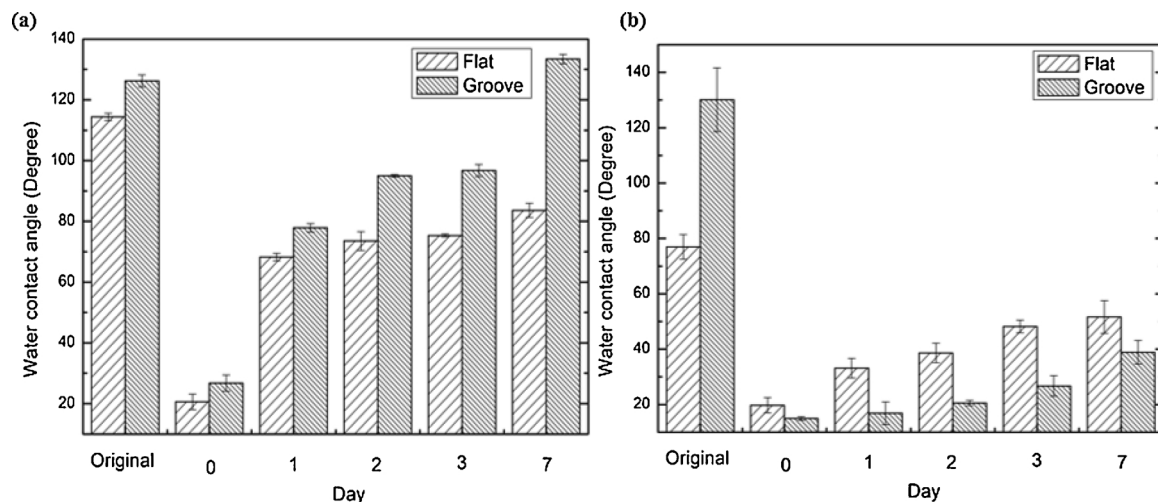


Fig. 4. Comparative water contact angles between (a) flat ($\sim 114.4^\circ$) and patterned PDMS ($\sim 126.2^\circ$) cantilevers, and (b) flat ($\sim 76.94^\circ$) and nanotextured PI ($\sim 130.10^\circ$) cantilevers.

$130.10^\circ \pm 11.50^\circ$, respectively. The large contact angle difference between the PDMS and PI materials, even with flat surfaces, is expected to have a significant influence on cell adhesion. The important point here is that the water contact angle is significantly increased when the nanotextures are formed on the both surfaces, however the inherent surface energies of both materials are quite different. In other words, the adhesion of the cardiomyocytes to the substrate is much better in the case of PI. This property of the material is considered to be a very important requirement in cell-related experiments.

Oxygen plasma treatment (80 W, 30 s) was performed on both the PDMS and PI cantilever surfaces because the hydrophobic properties of these materials are not conducive to cell attachment [30]. The nanotextured PDMS and PI cantilever surfaces became hydrophilic immediately following oxygen plasma treatment. However, the hydrophobic nature of the PDMS cantilever surfaces recovered more rapidly

over time, as indicated by the contact angle measurements in Fig. 4(a). Additionally, the recovery times and the water contact angles for PDMS and PI cantilevers with and without nanotextures were significantly different. On day seven after oxygen plasma treatment, the nanotextured PDMS cantilever completely recovered its original hydrophobicity, whereas the flat PDMS cantilever recovered only 72% of its original hydrophobicity. Therefore, on the plasma-treated flat PDMS cantilever, seven days after cell seeding, a considerable number of cardiomyocytes were still attached to the PDMS surface. In contrast, a considerable number of cardiomyocytes had already detached from the nanotextured surfaces after seven days. In the case of the cantilevers fabricated from PI material, the water contact angle increased slightly over the seven days following cell seeding, but the final water contact angle was still less than 60° , as shown in Fig. 4(b). The nanotextured PI cantilevers that were not subjected to oxygen plasma treatment also

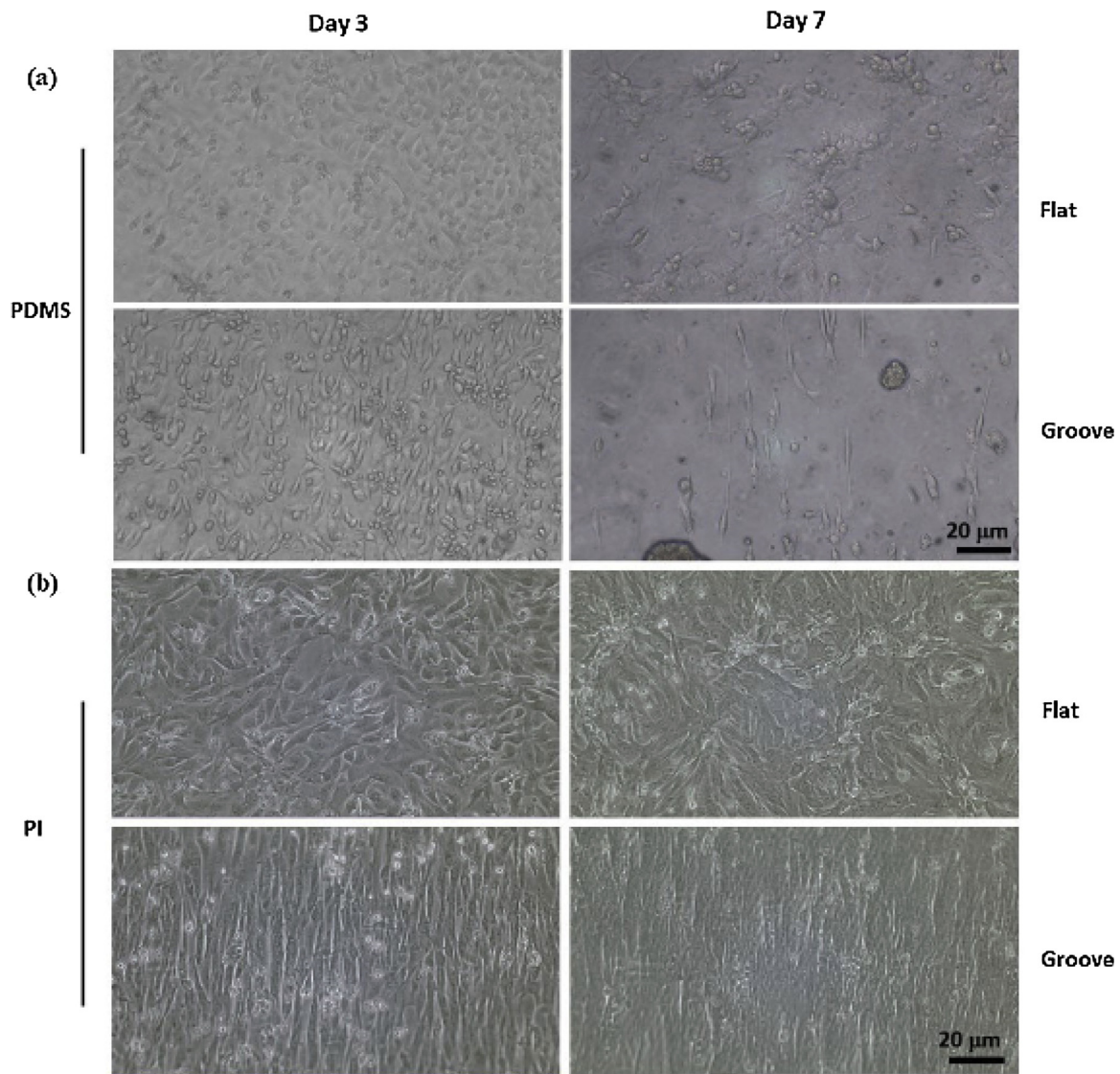


Fig. 5. Optical images of cardiomyocyte growth on the (a) flat and nanotextured PDMS, and (b) flat and nanotextured PI cantilevers.

exhibited remarkably high contact angles. However, the oxygen-plasma-treated PI cantilevers with nanotextures maintain contact angles lower than that of the PI substrate without nanotextures. This is presumably caused by the fact that the contact state of the water drops changes from the Cassie-Baxter model state to the Wenzel's model state based on the hydrophilic properties of the PI itself. Increased surface area with roughness is also effective in promoting adhesion and growth of cardiomyocyte. The chemical composition of the two different substrate materials before and after oxygen plasma treatment was confirmed by X-ray photoelectron spectroscopy (XPS, ESCALab Mark II, VG Scientific Ltd.) analysis as shown in Fig S5. The adhesion characteristics of cardiomyocytes cultured on the surface of the nanotextured PDMS and PI cantilevers were analyzed using the immunohistochemistry staining as shown in Fig S6 (Day 3 from NRVM process). The difference in the number of cardiomyocytes attached to the surfaces as well as the shape of the nucleus was clearly observed. Further, the adhesion characteristics on the nanotextured PDMS surface becomes worse as the incubation time increases, while it is maintained on the nanotextured PI surface.

3. Results & discussion

Fig. 5 presents optical images of cardiomyocytes on flat and

patterned cantilevers fabricated from PDMS and PI on days three and seven after cell seeding. From the day the cells began to grow together, the cardiomyocytes on the nanotextured PI cantilevers showed much better alignment and faster growth than those on the PDMS cantilevers or flat PI surfaces. In the case of the PDMS cantilevers, cardiomyocytes were often detached from the cantilever surfaces after seven days and beats became infrequent. In contrast, the cardiomyocytes grown on the PI cantilevers showed good contractility and cell junctions, even after seven days, on both the flat and nanotextured surfaces (Fig. S7). These results can be attributed to the different surface energies and stiffness values of the polymers used in cantilever fabrication [36,37]. Additionally, it was observed that the cardiomyocytes grown on the nanotextured PI cantilevers exhibited superior characteristics in terms of maturation and contraction force.

Immunofluorescence experiments were performed on day seven to compare and analyze the distributions and locations of the cardiomyocytes on different types of cantilevers. First, α -sarcomere actinin (constituting a Z-line) and the connexin-43 protein (cell junction protein) were stained. The sarcomere lengths for the flat and nanotextured PI cantilevers were $1.86 \pm 0.02 \mu\text{m}$ and $2.03 \pm 0.03 \mu\text{m}$, respectively. These values were analyzed based on expression of the α -sarcomere actinin protein, as shown in Fig. 6(a). The sarcomere length of the nanotextured PI cantilever increased by approximately 8% compared to

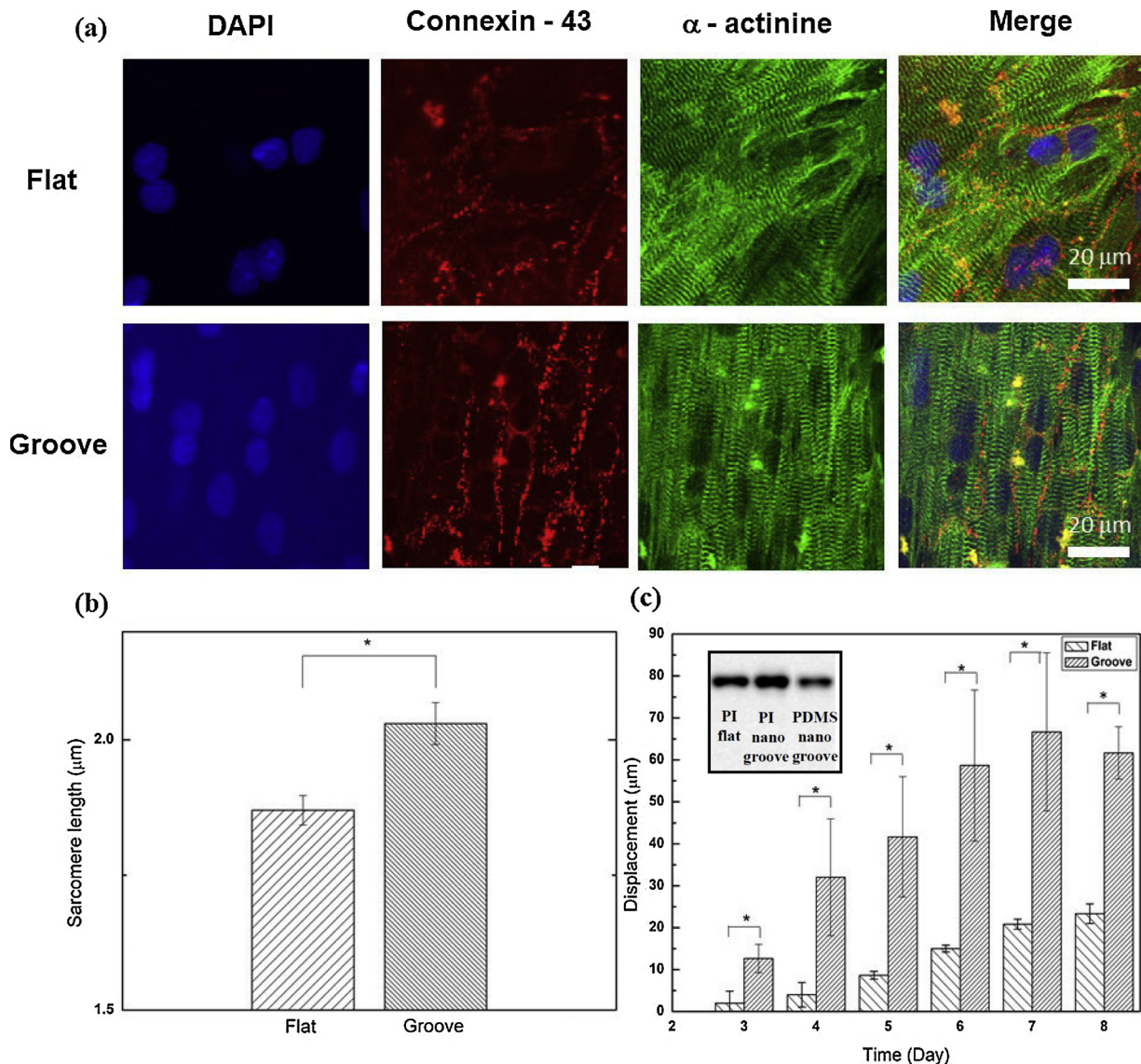


Fig. 6. (a) Immunohistochemistry staining of cardiomyocytes cultivated on the PI cantilevers, (b) sarcomere length (flat and groove samples have average lengths of $1.83 \pm 0.01 \mu\text{m}$ and $2.00 \pm 0.21 \mu\text{m}$, respectively ($n = 10$)), and (c) changes in cantilever displacement from day three to day eight. The cantilever displacements on day seven for the flat and nanotextured PI cantilevers are $20.83 \pm 1.17 \mu\text{m}$ and $66.66 \pm 18.85 \mu\text{m}$, respectively ($n = 3$).

that of the flat PI cantilever, as shown in Fig. 6(b). The sarcomere lengths of the cardiomyocytes on the nanotextured PI cantilever are over 90% of those of native cardiomyocytes in a living body [38]. The sarcomere lengths are also expressed in the direction of cardiomyocyte growth. Random growth and anisotropic growth of cardiomyocytes are clearly visible on the flat and nanotextured PI cantilever surfaces, respectively. Additionally, the cardiomyocytes on the PI cantilevers affect the displacement of the cantilevers. The displacement of the nanotextured PI cantilevers (68.6 ± 8.5) was approximately 2.4 times greater than that of the flat PI cantilevers ($28 \pm 4.3 \mu\text{m}$) on the seventh day after cell seeding, as shown Fig. 6(c).

Various information related to the beating frequency and contractile force of cardiomyocytes can be used as an index for the maturation of cardiomyocytes. In particular, immature cardiomyocytes exhibit low numbers of I_{K1} ions and cause spontaneous beating, whereas mature cardiomyocytes exhibit quiescent beating based on an increased density of I_{K1} ions [39]. On the seventh day after cell seeding, the spontaneous heart rates of the flat and nanotextured cantilevers were 54 ± 5 beats/min and 29.6 ± 2 beats/min, respectively, as shown in Fig. 7(a) and (b). Quantitative analysis of cardiac contractility and beating cycles

that vary with the drug concentration is considered to be an important factor of evaluating toxicity in the development of new drugs. Additional experiments were performed using two different cantilevers with flat and nanotextured surfaces. Verapamil is known to reduce contractility of cardiomyocytes with the increase of drug concentration. Therefore, the heart rates of the cardiomyocytes as a function of drug concentration were experimentally evaluated using the fabricated PI cantilevers and differences in response to Verapamil were analyzed using a LabVIEW-based analysis system, as shown in Fig. 7(c) and (d). The contractile force of the cardiomyocytes decreased to $93.15 \pm 4.39\%$ for the flat cantilever and $81.47 \pm 2.04\%$ for the nanotextured cantilever, respectively, with the addition of 1000 nM of Verapamil, as shown in Fig. 7(e) and (f). It is known that high doses of Isoproterenol, which is used for the treatment of bronchial asthma, respiratory tract disorders, and heart conduction disorders, can cause heart attack as a side effect. The experimental result of cardiomyocyte contraction according to the different concentration of the drug is shown in Fig. S8. According to the results, there was no change in the contractile force with increasing concentration of the Isoproterenol, however the change of beating cycle was very significant at the

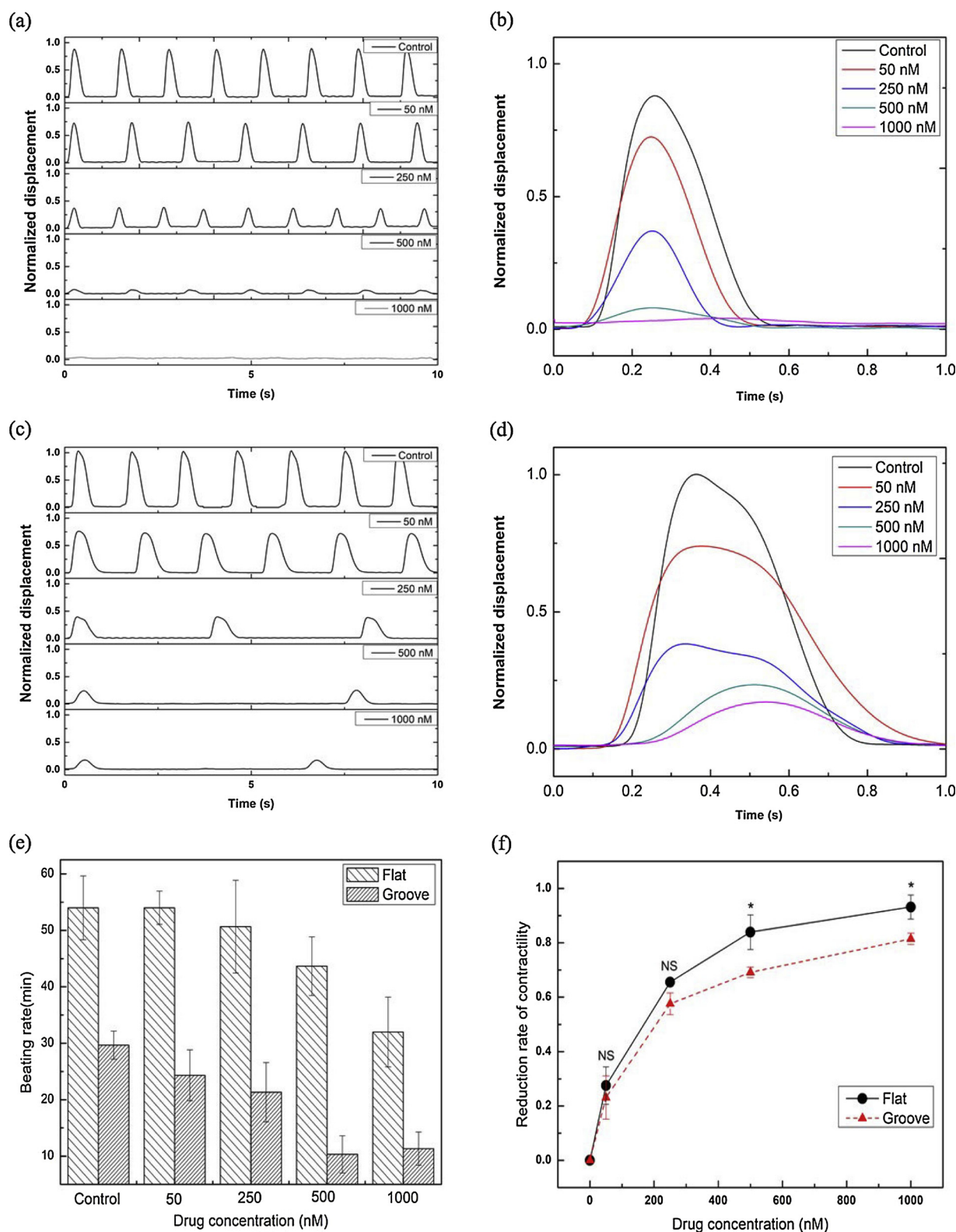


Fig. 7. Mechanical responses of cantilevers affected by Verapamil-treated cardiomyocytes: (a, b) flat and (c, d) nanotextured PI cantilevers. (a, c) show changes in beating frequency after treatment with various concentrations of Verapamil. (b, d) show single-cycle displacement at various concentrations of Verapamil (50 nM, 250 nM, 500 nM, and 1000 nM). (e) beating rate versus Verapamil drug concentration and (f) reduction in contraction rate with increasing Verapamil concentration (n = 3).

concentration of over 200 nM. In addition, the duration of a single beating was also greatly reduced at the same concentration.

The cardiomyocytes cultured on flat cantilevers showed a more sensitive response to the Verapamil by approximately 12%. This result

indicates that immature cells are more sensitive to drugs [40,41]. Immature cardiomyocytes are dependent on the hERG channel for repolarization and reaching their maximum diastolic potential. In contrast, the high ionic concentration of I_{K1} in mature cardiomyocytes is

reported to be less sensitive to hERG blockage [42]. Therefore, we can conclude that immature cardiomyocytes tend to be more sensitive in the hERG channel. Similar to previous studies, the mechanical behavior of the cardiomyocytes on the nanotextured cantilevers in our study showed more mature cardiomyocyte trends. Additionally, the use of a cantilever device not only facilitates parallel operation of the cantilevers, as shown in Fig. S9, but also simplifies the measurement system. PI cantilever arrays integrated with strain sensors can further increase the efficiency of drug toxicity analysis and will be studied in the future.

4. Conclusions

In this paper, we proposed a novel cantilever material and fabrication method to grow mature cardiomyocytes over a month by forming nanotextures on the cantilever surfaces. The nanotextured PI cantilevers are more stable than PDMS cantilevers in terms of cardiomyocyte adhesion and growth. Additionally, the nanotextures can be fabricated easily by using a conventional stamping technique, which provides low cost and high efficiency for mass production. Cardiomyocytes were grown on the proposed nanotextured PI cantilevers and improved contractile force was measured as a function of growth time. We also evaluated the drug-induced cardiac toxicity of Verapamil by using two different PI cantilevers with and without nanotextures. Different responses to the drug based on differences in contractility and growth of cardiomyocytes were experimentally confirmed. We believe that the nanotextured PI cantilevers affect cell maturation and they are expected to improve the accuracy of contraction-based drug toxicity testing in the near future.

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Appendix A. Supplementary data

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