BIOHYBRID MUSCLE FIBERS INTEGRATED IN A THREE-DIMENSIONAL CELLULAR CONSTRUCT

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ABSTRACT

We propose a method to construct a biohybrid tissue-like structure. In this structure, muscle fibers are fixed at several poles and maintain their tensions, and gold electrodes are located at the edges of the muscle fibers. Therefore, we can exercise contractions of the specific muscle fibers via the electrodes. Since a device with muscle fibers and electrodes is free-standing, it can be integrated in three-dimensional (3D) *in vivo*-like environments composed of living cells and extracellular matrixes (ECMs). We believe that our method is useful for fabrication of 3D hierarchic muscular structures for the evaluations of muscle behaviors in *in vivo*-like conditions.

KEYWORDS

muscle, flexible electrode, 3D structure, biohybrid device, parylene.

INTRODUCTION

A muscle fiber is a microsized linear actuator. When they are stimulated electrically, voltage-dependent Ca^{2+} channels are opened to introduce Ca^{2+} from the sarcoplasmic reticulum into the cytosol, and the Ca^{2+} influx induce contraction of muscle fibers [1]. Moreover, the power of the contraction depends on quantity of electricity and electrical pulse time, and the contractions are synchronized with electrical stimulations [2]. Owing to their controllable contraction and their high energy-conversion efficiency [3], reconstructed muscle fibers *in vitro* are useful for not only tissue engineering but also soft-robotics. Recently, many researchers have proposed devices combining muscle fibers with micro structures [4, 5]. However, it is difficult to apply stimuli to specific muscle fibers when they are packaged in other components because electrodes are not connected to each muscle directly.

Here, we propose a method to produce a free-standing device that muscle fibers are integrated with flexible electrodes. In the device, the muscle fibers kept their configuration and tension because they are fixed at the several poles on the substrate; cell culture under high tension is important for various muscular functions including a contraction of skeletal muscle fibers [6]. In addition, we can embed the device with muscle fibers in ECM and confined by layer of other cells. Since this device has electrodes, we stimulate the muscle fibers electrically via the electrodes even when they are packaged in ECMs and cells.

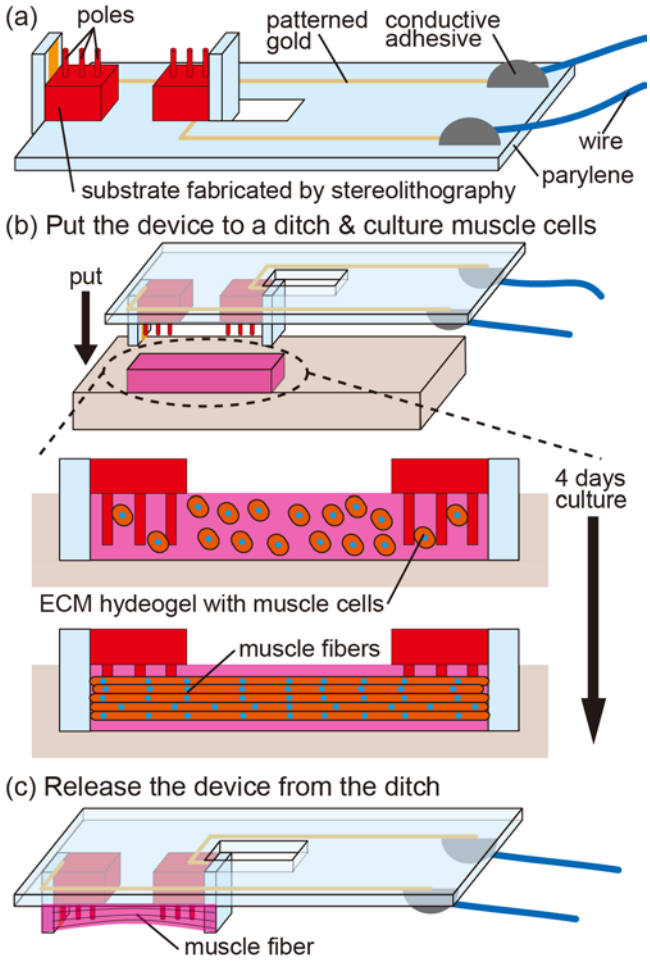

Figure 1: (a) Schematic diagram of the device with flexible electrodes. In the device, there are poles to fix edges of muscle fibers, and wires to electrically stimulate the muscle fibers. (b,c) Fabrication flow of producing the device with muscle fibers and flexible electrodes. (b) Putting the poles of the device into a ditch filled with ECM hydrogel with muscle cells. (g) After 4 days culture, we release the device with muscle fibers and electrodes from the ditch..

EXPERIMENT

1. Materials

Dulbecco's modified eagle's medium (DMEM), phosphate buffered saline (PBS), horse serum (HS) were purchased from SIGMA-Aldrich. Fetal bovine serum (FBS) was purchased from Japan Bioserum Co. Ltd. Other chemicals were purchased from Kanto Chemical Co., Inc and Wako Pure Chemical Industries.

A growth medium for muscle cells are composed of DMEM with 10% (v/v) FBS, 100 U/mL

penicillin and 100 μg/mL streptomycin. Meanwhile, a differentiated medium for muscle cells are composed of DMEM with 2% (v/v) HS, 100 U/mL penicillin and 100 μg/mL streptomycin.

We used parylene (Parylene C) as a material of flexible electrodes. Parylene is appropriate to be a base of the flexible electrodes (thickness: 12 μm) because parylene has a biocompatibility, workability, and flexibility.

The poles to fix edges of muscle fibers were fabricated by stereolithography machine (Perfactory, Envision Tec.). The resin used in stereolithography consists of acrylic oligomer, dipentaerythritol pentaacrylate, propoxylated trimethylolpropane triacrylate, photoinitiator and stabilizers. The poles were coated by parylene to improve their biocompatibility. We used a photocrosslikable glue (LOCTITE 3301, Henkel) when we bonded the poles on a parylene sheet.

2. Cell culture

To prepare muscle cells, we dissected hind limbs of rats (neonatal days 1-2). After a treatment of type-II collagenase to dissected hind limbs, primary rat myoblasts are collected by centrifugation.

All rats dissected in our experiments were maintained in accordance with the policies of the University of Tokyo Institutional Animal Care and Use Committee.

3. Fabrication method

To fabricate the device with muscle fibers and electrodes, we first produced a parylene sheet with patterned gold lines on a glass plate by standard photolithography, and peeled off the sheet from the plate. After bonding wires and substrates with poles on the sheet, we selectively patterned parylene on the sheet to make no parylene coated areas; at these areas, gold electrodes are exposed to be current-carrying parts. By cutting and folding the no parylene coated areas, we bonded the areas on the side wall of each substrate. Consequently, we obtained a flexible device with electrodes (Fig. 1(a)). For sterilization of the device, we dipped it in ethanol, dried it in a clean bench, and exposed it to ultraviolet lights over 1h. Subsequently, we prepared a poly(dimethylsiloxane) (PDMS) mold with same height as the poles, and put the poles of the substrates to a ditch filled with ECM hydrogel and muscle cells (Fig. 1(b)). Muscle cells in the ECM hydrogel differentiated into muscle fibers after 1 day culture in growth medium and 3 days culture in differentiated medium. The muscle fibers were tangled in the poles to fix themselves. Finally, we released the device from the ditch and obtained free-standing devices with muscle fibers and flexible electrodes (Fig. 1(c)).

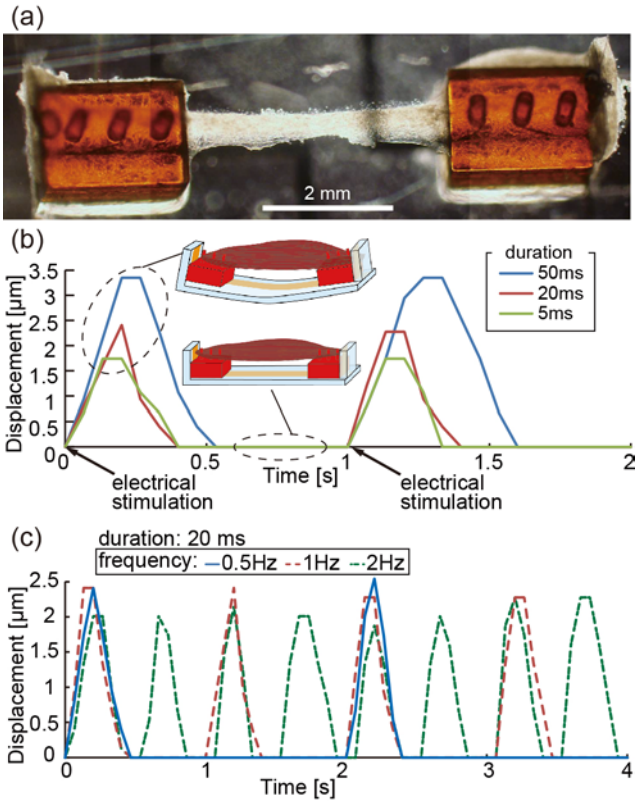

Figure 2: (a) Image of the device with muscle fibers and flexible electrodes. (b, c) Contraction displacement of the device with muscle fibers and electrodes by electrical stimuli ((b) electric field: 7.5 V/mm, (c) electric field: 7.5 V/mm, duration: 20 ms).

RESULTS AND DISCUSSION

Figure 2(a) shows the device with muscle fibers and flexible electrodes. The free-standing muscle fibers were fixed at the poles. Therefore, the muscle fibers kept their configuration on the device.

To evaluate the muscle fibers of the device, we transmitted electrical stimulations via the gold electrodes and observed bending of the device by contractions of the activated fibers. Figure 2(b, c) shows that the contractions of the muscle fibers coincided with electrical stimulations and changed according to the amount of stimulus time. This result shows that the muscle cells were differentiated into muscle fibers in the ditch of PDMS, and they have skeletal muscle properties [6]. Thus, these results indicate that the curvature of the device and the contraction of the muscle fibers are controlled by amount of electrical stimulations.

To demonstrate the ability of the package, we firstly embedded the device with muscle fibers and electrodes in collagen using a molding method. After gelling collagen and uncovering a mold, we cultured 3T3 cells on the collagen. Finally, the device with muscle fibers and flexible electrodes was confined by the collagen and a layer of 3T3 cells, resulting construction of a hybrid tissue-like structure. In the structure, we confirmed that almost all of 3T3 cells and muscle fibers are alive. Since the molding method facilitates integrating samples into arbitrary shaped casting gel, we can package the device into complex shaped collagen covered with 3T3 cells. We believe that our method is useful not only to produce biohybrid tissue-like structure, but also to assay muscle fibers in 3D tissue-like environments and to fabricate structures combined with organic and inorganic materials.

CONCLUSION

We realized the device with the free-standing muscle fibers and the flexible electrodes, and contraction of the device by electrical stimulations via the flexible electrodes. In addition, we succeeded in embedding the device into collagen gel covered by 3T3 cells. These results indicate that the device have potentials to become bio-actuators with complex structure and hierarchic configuration. Thus, although the results in the manuscript are preliminaries, we believe that our method is useful for fabrication of 3D hierarchic muscular structures to evaluate muscle behaviors in *in vivo*-like conditions.

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