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ORIGINAL RESEARCH PAPER

# Accelerated myotube formation using bioprinting technology for biosensor applications

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**Abstract** Muscle-powered, biological, microelectro-mechanical system is promising for actuator and biosensor applications. Functional conjugation between the cells, tissues, and biomolecules to the microdevice is crucial for this application. Bioprinting as an enabling technology possesses the advantages of high throughput, digital control, and highly accurate delivery of various biological factors to the desired locations for numerous applications such as 3D tissue fabrication. We have now evaluated the feasibility of the precise placement of mouse myoblasts onto micro-sized cantilevers. The evenly aligned printed cells fused with each other and formed mature myotubes after only 4 days. In contrast, it took more than 14 days for randomly deposited cells to do so. The printed myotubes were functional and responded to the electrical stimulation synchronously. Furthermore, the integrated Bio-MEMS device responded to the chemical stimulation spontaneously which demonstrated the potential as a functional biosensor. The contractility of the system was recovered quickly after the removal of the chemical stimulation, which indicated the flexibility of this system and the recycling potential.

**Keywords** Bioprinting · Biosensor · C2C12 · Muscle contraction · Thermal inkjet

## Introduction

Biological microelectro-mechanical system (Bio-MEMS) devices conjugated with biological components are promising for the development of novel bioengineering microdevices, such as motors and actuators (Xi et al. 2005), heart pumps (Tanaka et al. 2007), and biosensors (Harms et al. 2006). Muscle cells have been widely used in these applications by generating force activated by actin–myosin motors regulated by excitation–contraction coupling (Bers 2002). These muscle-powered microdevices utilizing energy generated by biochemical reaction are promising to save energy, resources, and spaces (Asano et al. 2012). The C2C12 skeletal muscle cells possess the advantages of infinite proliferation and differentiation into multinucleated myotubes (Yaffe and Saxel 1977).

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As a well established cell line, the overall properties of C2C12 cells cultured and differentiated in vitro have been tested to closely mimic the properties of skeletal muscle in vivo (Miller 1990). Although C2C12 cells have been widely used to incorporate with bio-microdevices for many applications, it is important that the muscle cells and microdevices are consistently conjugated to produce reliable and reproducible results. The traditional methodology for Bio-MEMS fabrication is to manually seed cells on or into the microdevices (Fujita et al. 2010). The randomly deposited cells through this approach were observed uneven on the microdevice which further affected the cell proliferation and differentiation. Therefore, it is critical to incorporate a precise cell seeding technology to develop the Bio-MEMS constructs with consistent cell arrangement.

Bioprinting based on thermal inkjet printing technology as an attractive enabling approach for tissue engineering and many other applications has the advantages of high throughput, digital control, and highly precise placements of cells and biological factors to the targeted two-dimensional (2D) and three-dimensional (3D) locations (Wilson and Boland 2003). This technology has been proved to be cell friendly and led to many successes in tissue engineering and many other biological fields (Cui and Boland 2009; Cui et al. 2010, 2012a, b, c).

In this study, a Hewlett-Packard (HP) Deskjet 500 thermal inkjet printer was modified to precisely print and align C2C12 cells onto the tiny cantilevers at 300 dpi (85 μm) resolution. In order to control the cell proliferation and differentiation with minimal variations, we printed the same number of cells to evenly cover each cantilever of the microdevice. The function of the formed myotube/cantilever constructs were tested with electrical field and chemical stimulations. The hypothesis of this study was the bioprinted C2C12 cells could form myotubes and respond to the various environment stimulations as a functional biosensor.

## Materials and methods

### Materials

Mouse C2C12 muscle myoblast cell line was purchased from Sigma-Aldrich. Silicon-based cantilever microdevices were provided by Lifetime Tech (Hong Kong, China). Dulbecco's modified Eagle's medium (DMEM) was obtained from Mediatech (Manassas, VA). LIVE/DEAD viability/cytotoxicity kit and penicillin/streptomycin/glutamine (PSG) were purchased from Invitrogen. All other chemicals were from Sigma-Aldrich unless otherwise noted.

### Cell culture and bioink preparation

Mouse C2C12 myoblasts were cultured at 37 °C with humidified air containing 5 % $CO_2$ in DMEM supplemented with 10 % fetal bovine serum (FBS) and 1 % PSG. Culture medium was changed every 3 days and the cells were used at 80–90 % confluence.

Bioink was prepared by suspending C2C12 cells in sterile phosphate-buffered saline (PBS) solution at $8 \times 10^6$ cells/ml.

### Biopaper (cantilever) preparation

The silicon-based cantilever microdevice had 16 cantilevers in each chamber. Each cantilever was 800 μm long, 100 μm wide and 5 μm thick. Biopaper was cleaned using a plasma cleaner (Harrick Plasma, Ithaca, NY) for 20 min. The cleaned cantilevers were then rinsed with 70 % (v/v) ethanol followed by sterile water. The sterilized microdevices were coated with fibronectin solution (5 μg/ml) for 2 h before printing or cell seeding.

### Bioprinting and myotube maturation

The bioprinting platform was set up as previously described (Cui et al. 2010). The printer was sterilized using UV for at least 2 h before using. HP Deskjet pens with Fifty firing nozzles were rinsed with 70 % (v/v) ethanol for three times followed by sterile water. The pen was then filled with bioink and ready to print. The distance between the printhead and biopaper was set at 1–2 mm. Digital patterns representing the shape and size of the cantilevers were designed using Adobe Photoshop (Adobe Systems, San Jose, CA) and printed to deposit the cells onto the biopaper. The cell concentration in the bioink was adjusted to print one cell in each ink drop (Cui et al. 2010). The cantilever with printed cells was carefully transferred to ultra-low attachment, well plates (Corning, Lowell, MA). After 5 min incubation, differentiation medium consisting of DMEM supplemented with 2 % (v/v) calf

serum, 1 nM insulin (Invitrogen), and 1 % PSG was slowly added surrounding the biopaper until it was immersed in the medium. Medium was replaced every 2 days during the culture. The viability of the printed C2C12 cells was measured with Live/Dead Viability/Cytotoxicity assay 12 h after printing. Live cells were stained into green fluorescence by calcein AM and dead cells were stained with red fluorescence by EthD-1. The cell viability was calculated by dividing the number of live cells by the sum of live and dead cells. The manually deposited samples were prepared by carefully pipetting the non-printed bioink onto the surface of cantilevers in a petri-dish. The myotube formation on the cantilevers was observed by optical microscopy during the culture.

Electric stimulation of bioprinted myotubes

After 4 days in culture, the constructed bio-microdevice was immersed in differentiation medium and stimulated with electric field in a square chamber. The chamber was perfused with 37 °C humidified air containing 5 % $CO_2$ during the experiment. Electric pulses (2 V, 40 ms duration) were generated by a pulse generator and applied to the substrate at various frequencies to test the integration and function of the bioprinted myotube/cantilever constructs. The movement of cantilevers caused by the contraction of myotubes upon stimulation was detected and recorded by a customized laser detection system (Lifetime Tech).

Myotube responses to the exogenous factors

To demonstrate the potential of using this microdevice for biosensor applications, veratridine (VTD) at 5 μm was added to the myotube/cantilever construct under 1 Hz pulse stimulation. VTD is alkaloid neurotoxin on nerve and muscle membranes by causing persistent opening of the voltage-gated sodium channels (Catterall 1980). The changes of the myotube contractile were detected by laser. After 8 min of incubation, the introduced chemical was carefully washed away and fresh media was added to evaluate the recovery of the bio-microdevice.

## Results and discussion

Bioprinted myotube formation on cantilevers

The viability of printed C2C12 cells was 91.2 ± 2.6 % ($n = 3$) and there was no significant difference in cell viability during the culture. The printed cells aligned

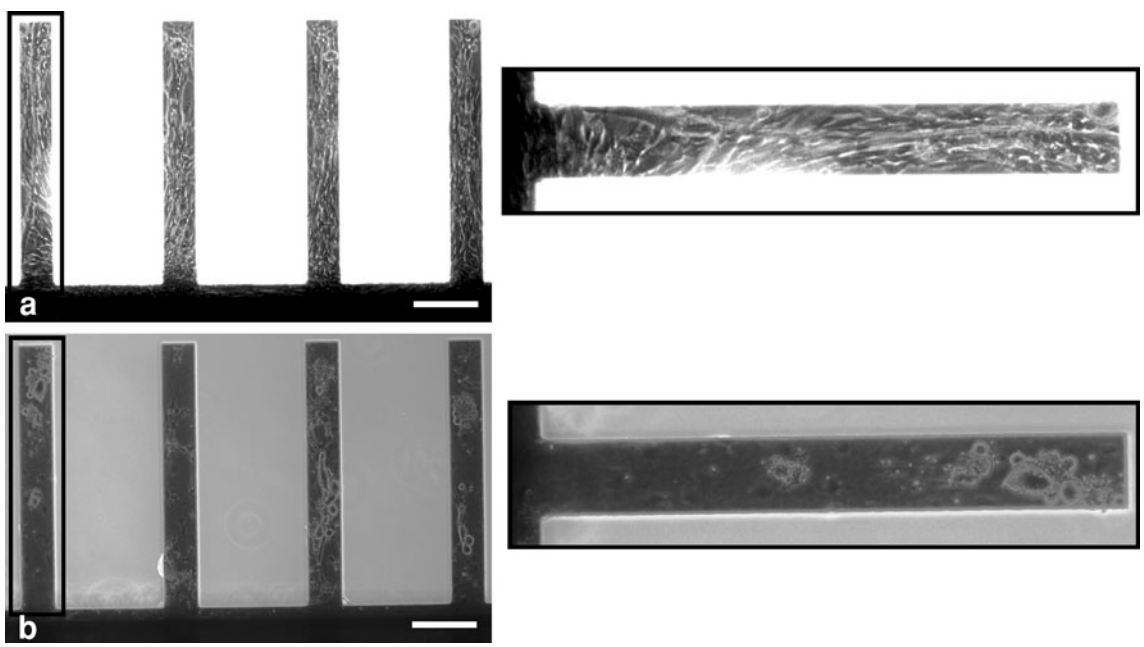



**Fig. 1** C2C12 cells on cantilevers. **a** Bioprinted cells formed myofibers on all cantilevers after 4 days in culture. **b** Non-printed cells randomly distributed on cantilevers and no myofibers were observed after 7 days. *Scale bars* 200 μm

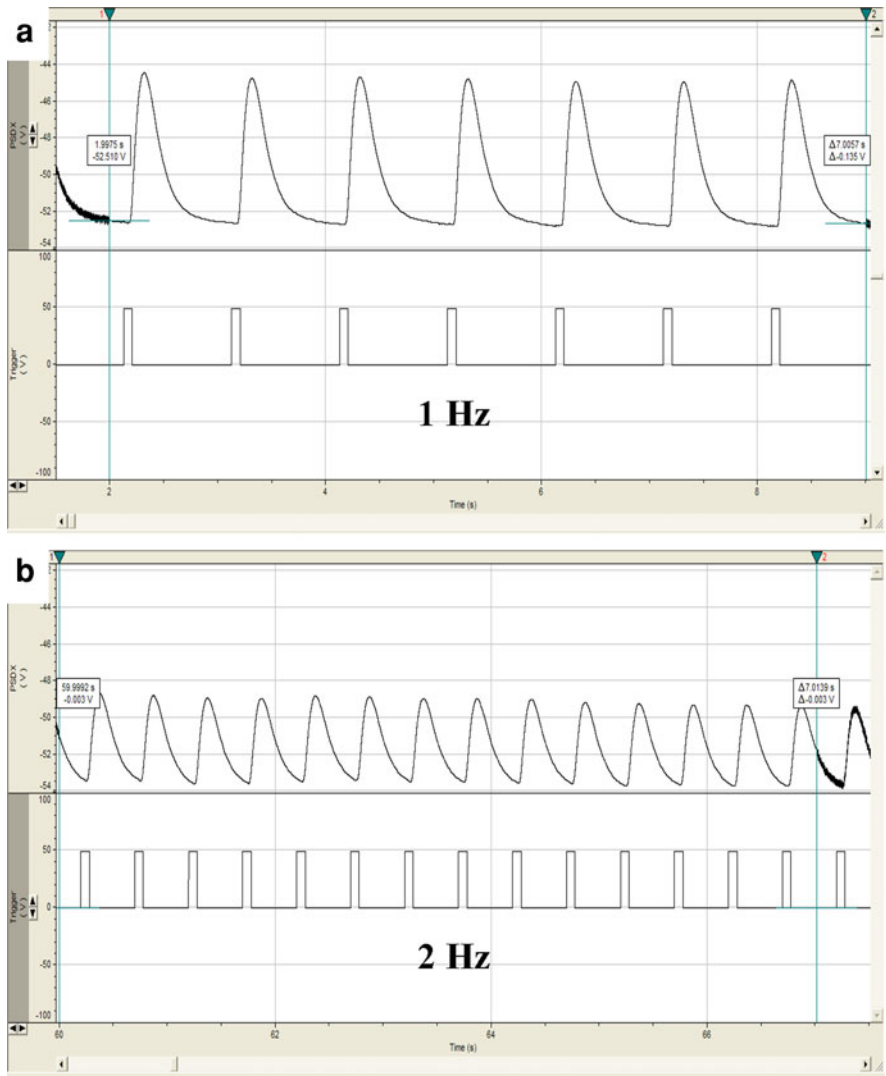


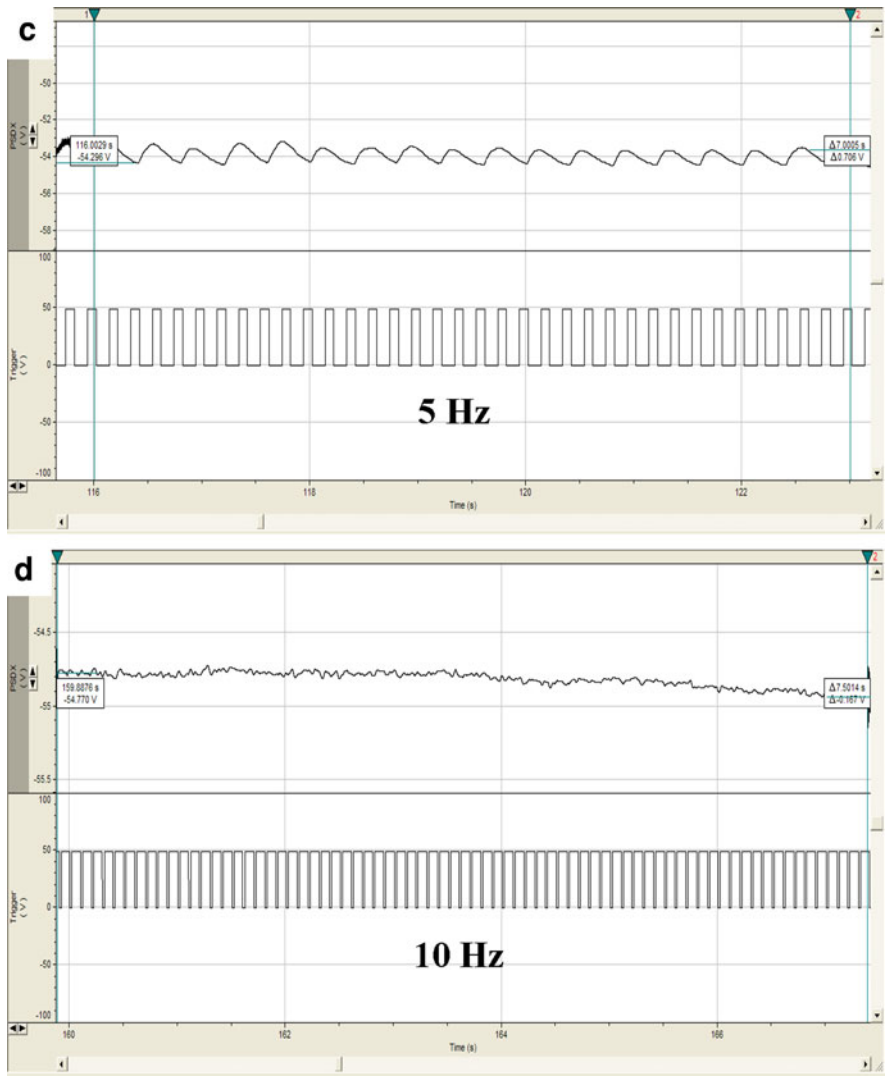

**Fig. 2** Myofiber contractions upon electric stimulation with 2 V and 40 ms duration for each pulse. **a** Myofiber contraction upon electric stimulation with 1 Hz pulse. **b** Myofiber contraction upon electric stimulation with 2 Hz pulse. **c** Myofiber contraction upon electric stimulation with 5 Hz pulse. **d** Myofiber contraction upon electric stimulation with 10 Hz pulse

closely with each other and formed confluent myotubes on almost all the cantilevers (97.3 ± 3.7 %, $n = 5$) after 4 days in culture (Fig. 1a). By contrast, there was no confluent myotube formation on the manually deposited cantilevers after 7 days in culture, probably due to the random and uneven cell coverage on the cantilevers initially (Fig. 1b). A few cantilevers with confluent myotube coverage (15.8 ± 5.9 %, $n = 3$) from the manually deposited samples were observed after 14 days in culture and most cantilevers were observed without myotube formation (84.2 ± 5.9 %, $n = 3$). Therefore, the precise deposition of C2C12 myoblasts on cantilevers is crucial for accelerated myotube formation due to closely aligned cells, as well as the even cell coverage on the microdevice. Thus the full or maximum capacity of the chips can be achieved using bioprinting. Only a few cantilevers of the bioprinted sample observed without myotube formation was probably due to the misalignment of the printed patterns during the printing. This issue might be minimized by stabilizing the printing platform as well as the biopaper during the printing process.

## Myotubes contractile with electric field stimulation

Conjugated myotube/cantilever constructs were stimulated by the electric pulses of 2 V with 40 ms duration at 1, 2, 5 and 10 Hz. The contraction of the

**Fig. 2** continued




constructs was detected by a customized laser detection system (Fig. 2). The constructs responded to the electric stimulations synchronously up to 5 Hz. At 10 Hz stimulation, it was difficult to observe the synchronous responses due to the limitation of biological reactions. This showed the bioprinted biomicrodevices possessed equal or even better physiological properties comparing to the conventionally fabricated constructs in term of the spontaneous responses to the stimulation. In addition, the bioprinted myotubes can also be used for muscle exercise studies with electric stimulations at various frequencies, which demonstrates the versatility of this work (Fig. 3).

## Bioprinted Bio-MEMS as a biosensor

The myotube/cantilever construct responded immediately upon the VTD introduction during the 1 Hz electric stimulation. With vibrating for a couple seconds upon the introduction of the exogenous toxin, the construct lost the synchronous contraction and no further contractions were detected. After 8 min incubation with the toxin, the chemical was removed by replacing with the fresh incubation medium. Surprisingly, the construct regained the muscular physiologic properties and contracted synchronously according to the electric stimulation again. Although the amplitude was smaller than that before the toxin introduction,

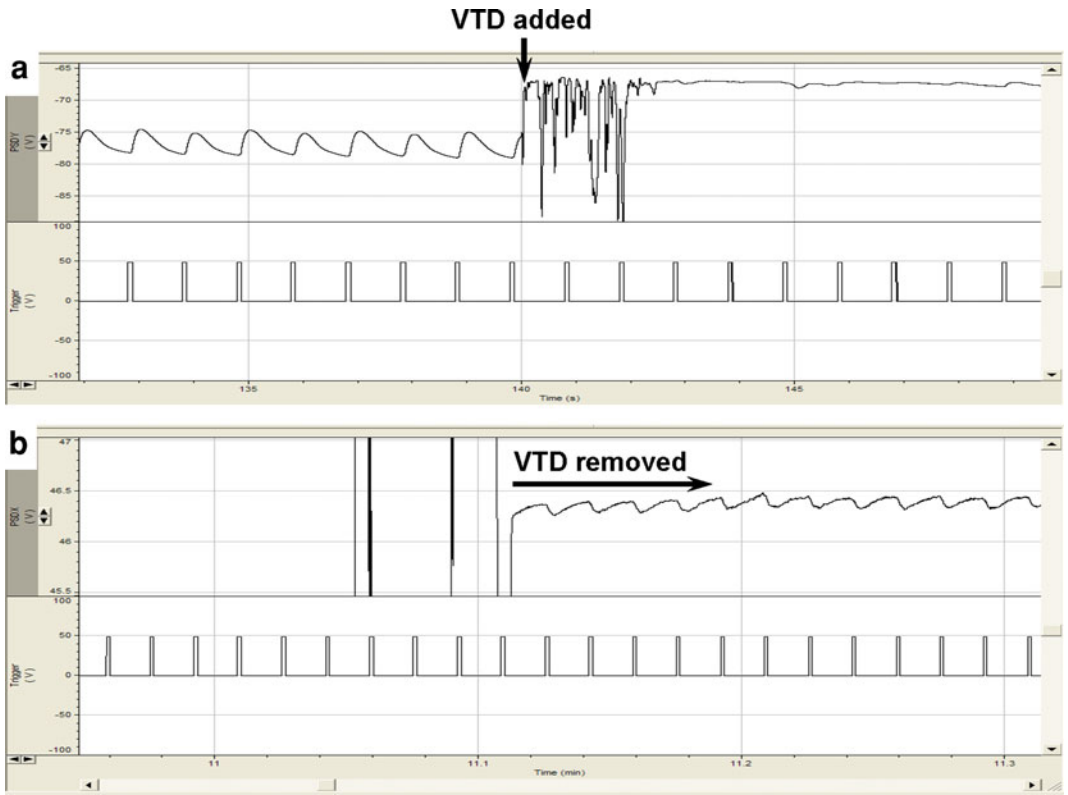



**Fig. 3** Contractile myofibers responded to the sodium channel agonist veratridine. **a** Veratridine at 5 μm was introduced to the contractile myofibers at 140 s during the recording. The muscle tissue lost the capacity to contract after 2 s. **b** After the toxin was removed, the same myofiber regain the ability of contraction synchronously with the electric stimulus

this still demonstrated the recyclable potential of using this construct for biosensor applications.

## Conclusion

We have developed a bioprinting platform for functional integration of cells and microdevices for biosensor application. Without complicated and expensive treatments of silicon-based microdevices, cells were precisely aligned to the targeted positions at high resolutions. The printed cells were alive and demonstrated robust proliferation and differentiation properties to form functional myotubes within only a few days in culture. The conjugated construct responded to the electric stimulation as well as exogenous chemical toxins spontaneously with reliable and reproducible properties. Furthermore, this Bio-MEMS device survived from the toxic treatment and recovered its physiological properties afterwards, which is promising as a recyclable biosensor.

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