MUSCLE-ACTUATED BIOMIMETIC HYDROGEL-BASED 3D MICROSKELETON

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ABSTRACT

This paper reports on a biomimetic microskeleton actuated by muscle fibers (figure 1(a)). The 3D microskeleton is based on concatenated rib elements fabricated by two-photon polymerization of a custom-made hydrogel (figure 1(b)). For the first time, micrometer-sized skeletons were fabricated, overgrown with functional muscle fibers, and contracted at significant amplitudes. Compared to planar biomimetic structures, 3D microskeletons can be shaped into arbitrary complex geometries, thereby opening pathways towards muscle-driven biohybrid microrobots.

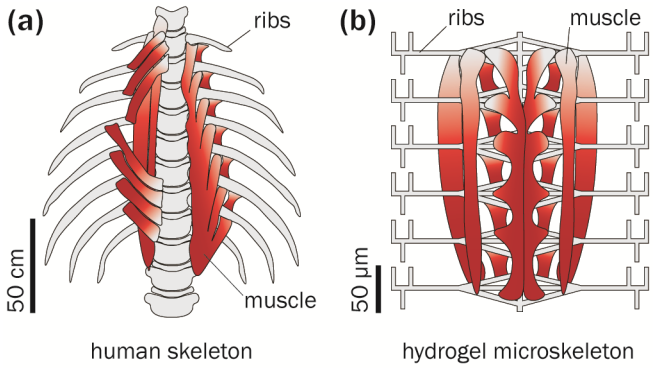

Figure 1: Schematic representation of the biomimetic microskeleton: (a) human skeleton model and (b) artificial hydrogel microskeleton.

INTRODUCTION

Due to its robustness, the C2C12 myoblast cell line is often targeted for bio-actuators in MEMS [1]. As the actuation force of C2C12 myotubes is between 0.5 and 1 µN they can deform gel-like materials. In previous muscle-driven actuators, the cells were therefore mostly cultured on millimeter-sized gel-like scaffolds [2,3]. Typically, C2C12 myotubes can achieve actuation amplitudes in the micrometer range, which compared to the millimeter dimensions of the scaffolds is rather small. The rationale of this paper is that downscaling the cell-culture scaffolds would permit to achieve actuation amplitudes comparable to the scaffold feature size. However, micro-structuring soft gels into mechanically stable 3D microskeletons is challenging. Here we take up this challenge. Recent advances in two-photon polymerization enabled the fabrication of cell culture microscaffolds with gel-like stiffness [1]. Building on these advances, we designed a novel hydrogel-based photopolymer which we used to fabricate biomimetic microskeletons able to promote localized and aligned muscle growth.

MATERIALS AND METHODS

Design

The microskeleton consists of concatenated rib elements which protrude from the plane (figure 2(a)). Muscle fibers grown on such structures will not be attached to the substrate and will thus actuate only the microskeleton. Typically, the microstructures are written as a single 3 µm high and 1 µm wide line [1]. However, their width can be locally increased by rewriting the lines several times and consecutively shifting them laterally by 300 nm (figures 2(b)-(d)).

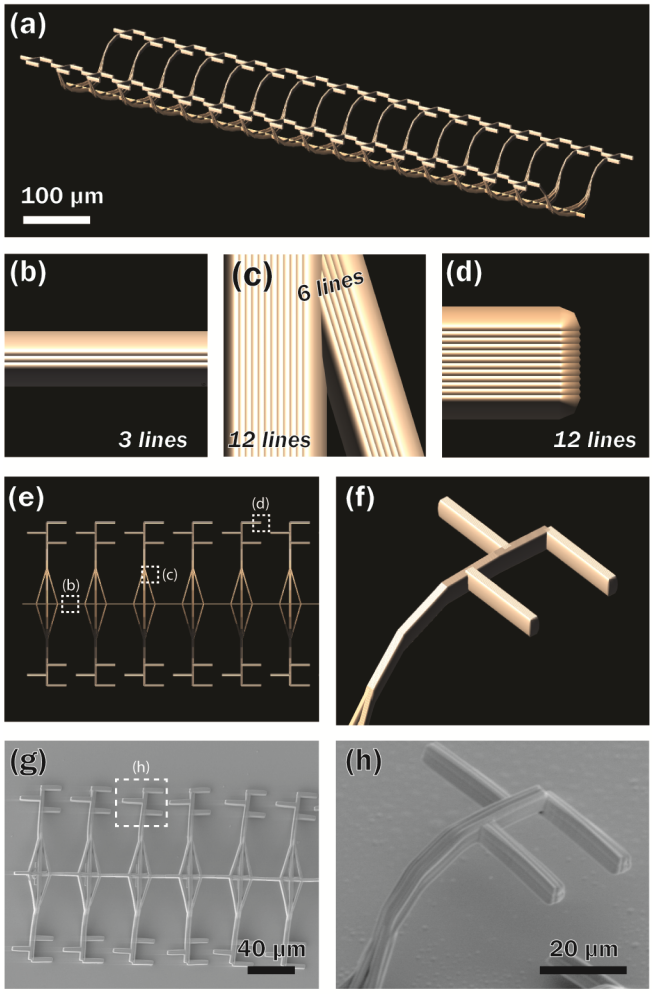

Figure 2: (a) CAD snapshot of the microskeleton rib structure; (b)-(d) Detailed view of the different structure widths from the regions highlighted in (e); (f) Detailed view of a suspended rib; (g) and (h) SEM micrographs corresponding to (e) and (f).

As described in our previous work [1] the successful differentiation of muscle cells into contractible muscle fibers requires a scaffold stiffness equivalent to a Young's modulus of 12 kPa which in our case corresponds to line a width of 2 µm [1]. The line structure connecting the rib elements shown in figures 2(b) and (e) were designed accordingly. The rib elements should not bend under the contraction of the muscle fibers and were therefore designed to be 5 µm wide (figures 2(c), (d)). In addition, the rib elements are laterally supported by 3 µm wide structures (figure 2(c)).

The comb structures at the endpoints of the ribs (figures 2(f), (h)) have two functions: First, the combs are spaced by 20 µm which is smaller than the typical cell diameter. When the cells are seeded onto the microskeleton, the combs will trap muscle cells. Cells can thereafter be cultured, while being suspended above the substrate. Second, the combs are designed to guide the cell fusion and to force the muscle fibers to align along the microskeleton [1].

Materials

The hydrogel used for the microskeleton consists of polyethylene glycol diacrylate (PEGDA, Sigma Aldrich) with additional 5% of pentaerythritol tri-acrylate (PETA, Sigma Aldrich). PETA provides additional acrylate groups which can be crosslinked and thereby increase the stability of the microstructure, thus preventing its swelling when immersed in aqueous solutions. Moreover, compared to pure PEGDA, the additional PETA provides sites which can be functionalized with cell adhesion proteins.

The muscle cells used are a C2C12 murine cell line (C3H muscle myoblast, Sigma Aldrich). The cell culture medium is high-glucose Dulbecco's Modified Eagle Medium with added 10% fetal bovine serum, whereas the differentiation medium is supplemented with 5% horse serum. All cell culture supplies are from Sigma Aldrich.

Microfabrication

The fabrication process described in figure 3(a) starts with acetone and isopropanol cleaned glass cover slides. Prior to silanization, the slides were oxygen plasma-activated (40 W, 5 min). Thereafter, hydrophilic polymer brushes (figure 3(a) inset (1)) based on poly-dimethyl acrylamide were applied by dip coating and covalently linked to the glass by UV curing [4]. The polymer brushes serve to prevent protein absorption and cell adhesion. The hydrogel is then drop-cast onto the glass cover slide. Two-photon laser polymerization (Nanoscribe, Germany) with a line writing speed of 90 µm/s and a laser power of 10 mW was used to pattern the microstructures into the hydrogel photoresist. The microskeletons were then revealed in two consecutive isopropanol baths for 15 min each and allowed to dry in air. Figures 2(g) and (h) show SEM micrographs of the skeleton at this stage. Finally, the devices are sterilized by rinsing in three consecutive 70% ethanol baths for 10 min each and are then kept for 12 h under UV irradiation.

An important property of cell scaffolds is their affinity to cell adhesion. To promote this property, the skeletons were functionalized in a 10 µM fibronectin solution (Biomedical Technologies Inc., USA) for 2 h and rinsed twice in phosphate-buffered saline solution (inset (2) of figure 3(a)) The scaffolds are then immersed in C2C12 cell culture media and stored inside a cell incubator at 37°C and 5% CO_2 atmosphere.

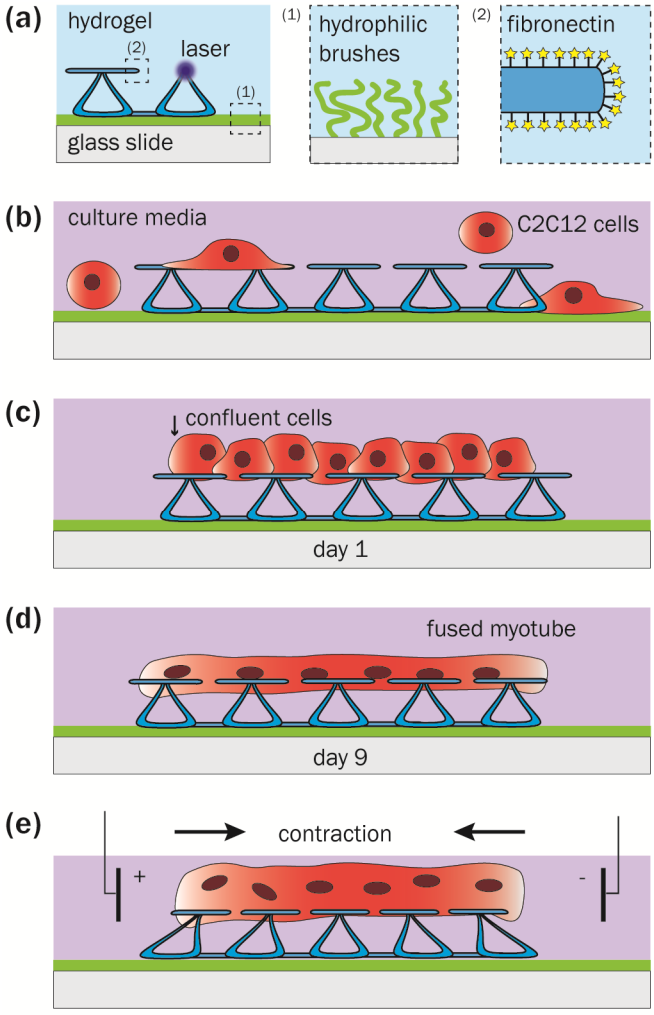

Figure 3: (a) Microfabrication of the microskeleton with details on the cell repellent coating (1) and cell adhesion promoting fibronectin coating (2); (b)-(d) Cell culture procedure; (e) Actuation.

Cell Culture

The C2C12 cells were cultured in petri dishes using the above-mentioned medium for at least two cell passages after thawing. This guaranteed a healthy cell population. To minimize cell damage or mutations due to long-term culturing, only cell cultures with fewer than nine passages were used. Once the C2C12 cells reached a confluency of 70%, they were released and reseeded into a new culture dish containing the microskeletons (figure 3(b)). The cells were seeded at a density of 10^4 cells/ml and reached full confluency within 24 h (figure 3(c)). The cell fusion and

differentiation was induced by switching to differentiation medium. During the following nine days of culture the cells fused and bundled into functional myotubes (figure 3(d)).

Actuation

The successfully differentiated myotubes were actuated by electrical depolarization of the cell membrane (figure 3(d)). To this purpose, the cell culture dishes were mounted into a cell culture stimulator (C-Pace EP, IonOptix, USA). Sterile carbon electrodes where placed 2 cm apart into the culture medium and centered on the microskeleton. The myotubes were then actuated by applying bipolar voltage pulses with a pulse width of 40 ms and amplitude of ±12 V. Cell damage was avoided by stimulating for 20 min and pausing for 10 min. After stimulation, the culture medium was replaced with fresh medium.

RESULTS AND DISCUSSION

Cell Growth and Differentiation

The differentiation of single C2C12 cells into mature muscle fibers happens in three steps (figure 4(a)). During the first five days, the initially separated cells fuse into thin muscle fibers. During the next two days, they progressively bundle into thicker fibers. At this stage the muscle fibers cannot contract. Two further days are necessary for the muscle fibers to differentiate and build the internal sarcomere structure able to contract the cell upon electrical stimulation. During this last step, the cell cores migrate into densely packed clusters. As explained above, sarcomeres are expressed only when the muscle fibers are allowed to differentiate on soft microstructures. Figures 4(b) and (c) show an optical micrograph and a fluorescence micrograph of immunostained sarcomeres of successfully differentiated muscle fibers.

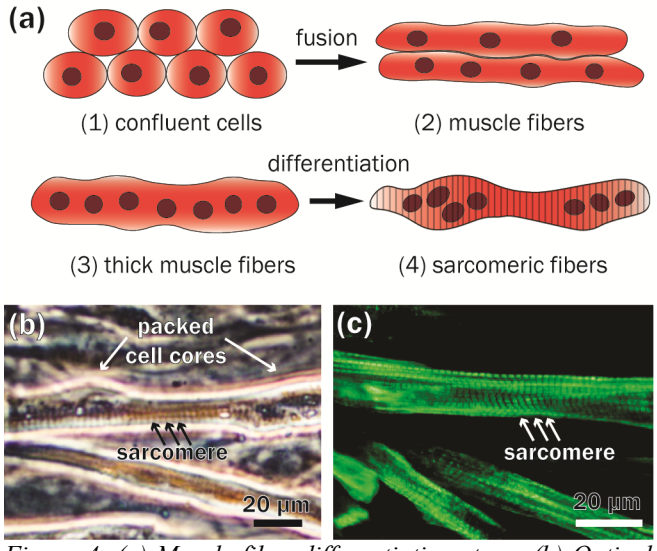

Figure 4: (a) Muscle fiber differentiation steps; (b) Optical micrograph of differentiated muscle fibers; (c) Fluorescence micrograph of immunostained sarcomeres.

The time lapse snapshots of the long-term culture and differentiation of C2C12 cells in figure 5 clearly demonstrate the localized growth and shape transition from randomly distributed muscle cells to axially aligned fibers. Although the glass substrates were previously coated with cell repellent polymer brushes, the cell adhesion promoter may still partially coat the glass surface at the bottom of the brushes. Initially the seeded cells can therefore partially attach to the glass substrate while growing to full confluency (figure 5(a)). During differentiation, the internal cell traction forces gradually increase. The weak adhesion to the glass becomes insufficient and the cells start to migrate towards the microstructure which provides strong cell adhesion properties (figures 5(b), (c)). The fully differentiated muscle fibers either specifically attached to the microskeleton, or detached and curled into cell clusters (figure 5(d)).

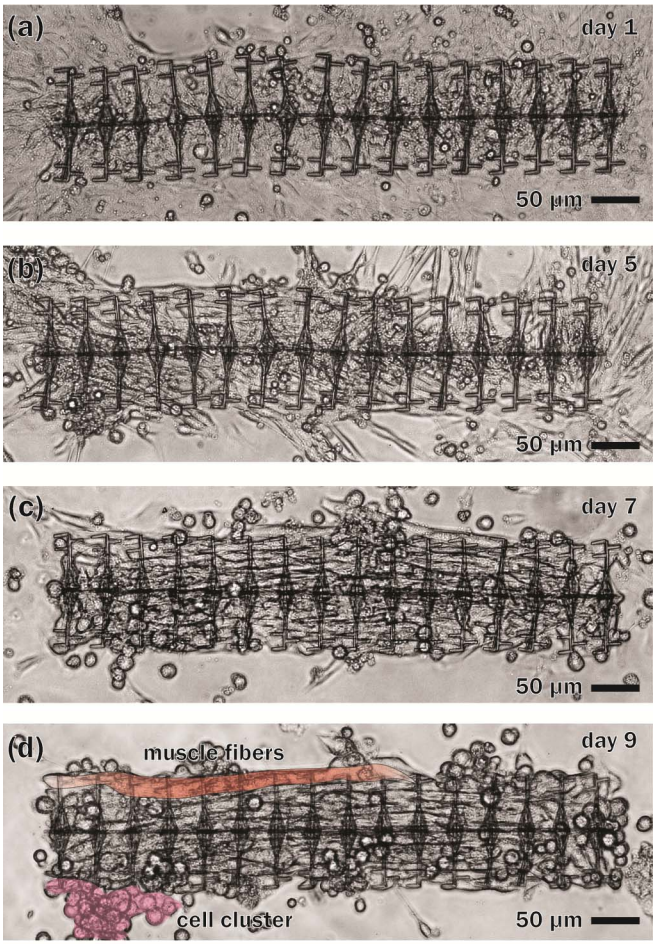

Figure 5: Time-lapse snapshots of cell culture: fusion, differentiation and migration from randomly to axially aligned muscle fibers.

During fusion, the myotubes kept an average aspect ratio between 1:8 and 1:13 (figure 6). We found this ratio to be a reliable indicator of successful differentiation into muscle fibers. As shown in figure 6, the absolute variation in fiber diameter increases with time. We observed that fibers which start to express sarcomeres at an early stage are unlikely to

continue to grow in diameter, in contrast with muscle fibers which have not yet expressed sarcomeres. After nine days of culture, 75% of the muscle fibers are found to contract upon electrical stimulation.

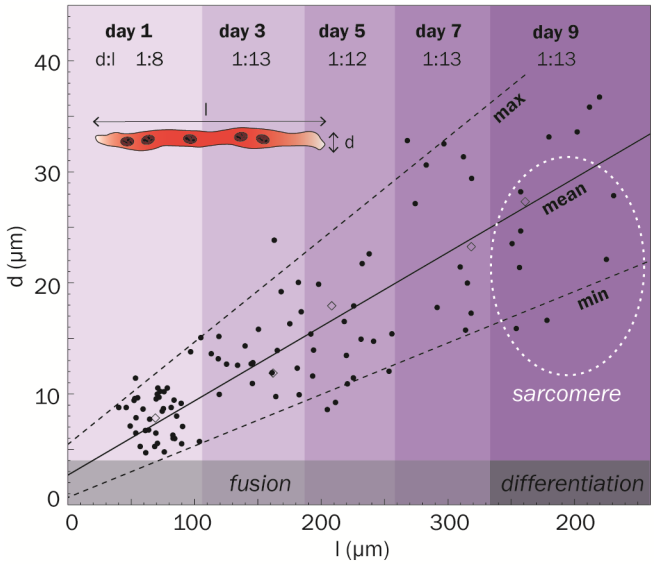

Figure 6: Ratio d:l of muscle fiber diameter d and length l during the differentiation.

Contraction

The muscle fibers successfully contract and deform the microskeleton under electrical stimulation (figure 7). Compared to previous work [1] where we used gold electrodes, the stimulation with carbon electrodes causes less hydrolysis known to increase the pH of the culture medium. Therefore, the microskeletons could be stimulated and trained over several hours. We achieved a maximal actuation amplitude of 12 µm which is an appreciable fraction of the microskeleton dimensions (figure 8). By stimulating at different frequencies, we were able to control the retraction distance of the muscle fibers (figure 8). At frequencies above 5 Hz, the microskeleton remained in the contracted state.

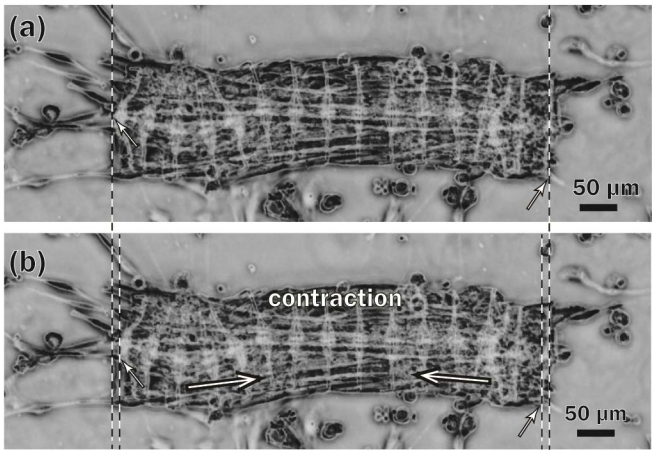

Figure 7: Phase contrast images of a microskeleton in (a) relaxed and (b) contracted state.

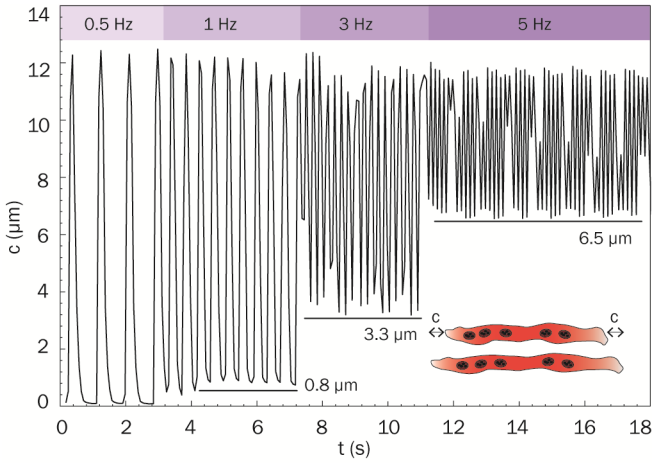

Figure 8: Contraction amplitude at different stimulation frequencies.

CONCLUSION

We successfully fabricated a biomimetic microskeleton overgrown with aligned and functional muscle fibers. The microskeletons were successfully actuated and the retraction distance could be controlled. These features are useful for the controlled locomotion of biomimetic microrobots.

ACKNOWLEDGEMENTS

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