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TITLE:

Bioinspired Soft Robot with Incorporated Microelectrodes

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SUMMARY:

A bioinspired scaffold is fabricated by a soft photolithography technique using mechanically robust and electrically conductive hydrogels. The micropatterned hydrogels provide directional cardiomyocyte cell alignment, resulting in a tailored direction of actuation. Flexible microelectrodes are also integrated into the scaffold to bring electrical controllability for a self-actuating cardiac tissue.

ABSTRACT:

Bioinspired soft robotic systems that mimic living organisms using engineered muscle tissue and biomaterials are revolutionizing the current biorobotics paradigm, especially in biomedical research. Recreating artificial life-like actuation dynamics is crucial for a soft-robotic system. However, the precise control and tuning of actuation behavior still represents one of the main challenges of modern soft robotic systems. This method describes a low-cost, highly scalable, and easy-to-use procedure to fabricate an electrically controllable soft robot with life-like movements that is activated and controlled by the contraction of cardiac muscle tissue on a micropatterned sting ray-like hydrogel scaffold. The use of soft photolithography methods makes it possible to successfully integrate multiple components in the soft robotic system, including micropatterned hydrogel-based scaffolds with carbon nanotubes (CNTs) embedded gelatin methacryloyl (CNT-GelMA), poly(ethylene glycol) diacrylate (PEGDA), flexible gold (Au) microelectrodes, and cardiac

muscle tissue. In particular, the hydrogels alignment and micropattern are designed to mimic the muscle and cartilage structure of the sting ray. The electrically conductive CNT-GelMA hydrogel acts as a cell scaffold that improves the maturation and contraction behavior of cardiomyocytes, while the mechanically robust PEGDA hydrogel provides structural cartilage-like support to the whole soft robot. To overcome the hard and brittle nature of metal-based microelectrodes, we designed a serpentine pattern that has high flexibility and can avoid hampering the beating dynamics of cardiomyocytes. The incorporated flexible Au microelectrodes provide electrical stimulation across the soft robot, making it easier to control the contraction behavior of cardiac tissue.

INTRODUCTION:

Modern state-of-the-art soft robots can mimic the hierarchical structures and muscle dynamics of many living organisms, such as the jellyfish[1,2], sting ray[2], octopus[3], bacteria[4], and sperm[5]. Mimicking the dynamics and architecture of natural systems offers higher performances in terms of both energetic and structural efficiency[6]. This is intrinsically related to the soft nature of natural tissue (e.g., skin or muscle tissue with a Young's modulus between 10^4–10^9 Pa) which allows for higher degrees of freedom and superior deformation and adaptability when compared with standard engineered actuators (e.g., a Young's modulus usually between 10^9–10^{12} Pa)[6]. Cardiac muscle-based soft-actuators, especially, show superior energy efficiency due to their self-actuation as well as their potential for autorepair and regeneration when compared to a mechanically based robotic system[7]. However, the fabrication of soft robots is challenging due to the necessity of integrating different components with different physical, biological, and mechanical properties into the one system. For example, engineered synthetic systems need to be integrated with living biological systems, not only providing them with structural support but also influencing and modulating their actuation behavior. In addition, many microfabrication methods require harsh/cytotoxic processes and chemicals that decrease the viability and function of any living components. Therefore, new approaches are necessary to enhance the functionality of the soft robots and to control and modulate their behavior.

To successfully integrate living components with good viability, a hydrogel-based scaffold is an excellent material to create the body of a soft robot. A hydrogel's physical and mechanical properties can easily be tuned to create microenvironments for living components such as muscle tissues[8,9]. Also, it can easily adopt various microfabrication techniques, resulting in the creation of hierarchical structures with high fidelity[1,2,10]. Flexible electronic devices can be incorporated into the soft robot to control its behavior with electrical stimulation. For example, optogenetic techniques to engineer electrogenic cells (e.g., cardiomyocytes), which show a light-dependent electrophysiological activation, have been used to develop a polydimethylsiloxane (PDMS)-based soft robotic sting ray guided by light that was able to recreate the undulatory movement of the fish in vitro[2]. Although optogenetic techniques have shown excellent controllability, the work presented uses electrical stimulation, a conventional and traditional simulation method. This is because electrical stimulation via flexible microelectrodes is easy and simple compared to optogenetic techniques, which require extensive development processes[11]. The use of flexible electronic devices can allow for long-term stimulation and standard/simple fabrication processes as well as tunable biocompatibility and physical and mechanical properties[12,13].

Here, we present an innovative method to fabricate a bioinspired soft robot, actuated by the beating of engineered cardiac muscle tissue and controlled by electrical stimulation through embedded flexible Au microelectrodes. The soft robot is designed to mimic the muscle and cartilage structure of the sting ray. The sting ray is an organism with a relatively easy to mimic structure and movement compared to other swimming species. The muscles are recreated in vitro by seeding cardiomyocytes on an electrically conductive hydrogel micropattern. As previously reported, incorporating electrically conductive nanoparticles such as CNT in the GelMA hydrogel not only improves the electrical coupling of the cardiac tissue, but also induces an excellent in vitro tissue architecture and arrangement[8,9]. The cartilage joints are then mimicked using a mechanically robust PEGDA hydrogel pattern that acts as the mechanically robust substrate of the whole system. Flexible Au microelectrodes with a serpentine pattern are embedded in the PEGDA pattern to locally and electrically stimulate the cardiac tissue.

PROTOCOL:

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the institutional Animal Care and Use Committee (IACUC) of Brigham and Women's Hospital.

1. GelMA synthesis

1.1. Dissolve 10 g of gelatin in 100 mL of Dulbecco's phosphate-buffered saline (DPBS) using a magnetic stirrer at 50 °C.

1.2. Add 8 mL of methacrylic anhydride slowly while stirring the gelatin prepolymer solution at 50 °C for 2 h. Dilute the reacted gelatin solution with preheated DPBS at 50 °C.

1.3. Transfer the diluted solution into dialysis membranes (molecular weight cutoff = 12–14 kDa) and place them into deionized (DI) water. Perform dialysis at 40 °C for about 1 week.

1.4. Filter the dialyzed GelMA prepolymer solution using a sterile filter (pore size = 0.22 μm) and transfer 25 or 30 mL of the solution into 50 mL tubes and store at -80 °C for 2 days.

1.5. Freeze-dry the frozen GelMA prepolymer solution using a freeze dryer for 5 days.

2. Preparation of poly(ethylene glycol) diacrylate (PEGDA) prepolymer solution

2.1. Dissolve 200 mg (20% of total solution) of PEGDA (MW = 1,000) with 5 mg (0.5% of total solution) of 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (photo-initiator, PI) in 1 mL of DPBS.

2.2. Incubate the prepolymer solution at 80 °C for 5 min.

3. Preparation of GelMA-coated CNT dispersed stock solution

3.1. Dissolve 80 mg of GelMA (used as a biosurfactant) in 4 mL of DPBS and then add 20 mg of COOH functionalized multiwalled carbon nanotubes (MWCNTs) and 0.5 mL of DI water into the GelMA prepolymer solution.

3.2. Sonicate the MWCNT-laden GelMA prepolymer solution for 1 h (0.66Hz, 100 Watt).

NOTE: During the sonication process, the solution must be immersed in a water bath at ~15 °C to prevent evaporation of solvent due to the rise in temperature.

4. Preparation of 1 mg/mL CNT containing 5% GelMA prepolymer solution

4.1. Dissolve 50 mg of GelMA and 5 mg (0.5% of total solution) of PI in 0.8 mL of DPBS at 80 °C for 10 min.

4.2. Add 0.2 mL of the prepared CNT stock solution (step 3). Vortex and incubate the solution at 80 °C for 10 min.

5. Preparation of a 3-(trimethoxysilyl)propyl methacrylate (TMSPMA) coated glass slide

5.1. Wash the glass slides (thickness = 1 mm, size = 5.08 cm x 7.62 cm) with pure ethanol.

5.2. Stack the cleaned slides vertically in a 250 mL beaker and spread 3 mL of TMSPMA on top of them using a syringe. Cover the beaker with aluminum foil to prevent evaporation of TMSPMA.

5.3. Incubate the slides in an 80 ˚C oven for 1 day.

5.4. Wash the coated glass slides by dipping them into pure ethanol, then dry.

5.5. Store the coated glass slides wrapped in aluminum foil at room temperature (RT).

NOTE: Try to minimize touching the surfaces of the TMSPMA-coated glass slides.

6. Fabrication of the flexible Au microelectrodes

6.1. Design a shadow mask using computer-aided design (Supplementary File 3).

6.2. Fabricate and purchase a shadow mask.

6.3. Wash the glass slide (thickness = 1 mm, size = 3 cm x 4 cm) with acetone and dry with a compressed air gun.

6.4. Attach the shadow mask to the glass substrates using double sided tape, then put them in an E-beam evaporator and wait until the chamber pressure reaches at least 10^{-6} Torr.

NOTE: The two pieces of tape were placed manually on the support at a distance short enough to host the glass and large enough to fit the entire pattern. This step takes around 45–60 min.

6.5. Deposit a 200 nm thick Au layer by E-beam evaporator (e.g., with Denton EE-4, vacuum = 10^{-6} Torr, power = 2.6%, rate = 2 Å/s) and cut the fabricated microelectrodes using a dicing saw machine (electrodes size = 7.38 mm x 8.9 mm x 200 nm).

7. Fabrication of an Au microelectrode-integrated micropatterned multilayered hydrogel scaffold

NOTE: The result of this procedure is a membrane where a micropatterned PEGDA hydrogel is in the bottom layer, a micropatterned CNT-GelMA hydrogel is on top, and the Au microelectrodes are between the two layers. This configuration ensures a better flexibility to the electrode and limits the risk of breaking.

7.1. Design and fabricate two photomasks to create the micropatterned PEGDA (1st photomask) and the CNT-GelMA hydrogel (2nd photomask) layers. See Supplementary File 2–3. The design can be done by using CAD software.

NOTE: See **Figure 2B,E**.

7.2. Pour 15 µL of 20% PEGDA prepolymer solution on top of the Au microelectrodes, then cover with the TMSPMA coated glass slide. Place the 1st photomask for the glass slide (micropatterned PEGDA) on top of the TMSPMA coated glass slide and expose the whole construct to UV light (200 W mercury vapor short arc lamp with 320–390 nm filter) at 800 mW of intensity and 8 cm distance for 110 s.

NOTE: See **Figure 1A**.

7.3. Add DPBS to surround the glass slide and detach the micropatterned PEGDA hydrogel together with the Au microelectrodes from the uncoated glass substrate carefully after 5–10 min to obtain the glass slide that has the micropatterned PEGDA hydrogel with the Au microelectrodes.

NOTE: See **Figure 1B**. Due to the TMSPMA coating, the construct is transferred from the uncoated glass substrate to the TMSPMA-coated one. Detach carefully because the Au microelectrodes can break easily during this step (**Figure 3**).

7.4. Place 100 µm spacers made by stacking two layers of commercial transparent tape (thickness = 50 µm) on the bottom of a Petri dish. Deposit a drop of 20 µL CNT-GelMA prepolymer solution between the spacers and then flip the glass slide obtained in 7.3 and fix it onto the dish with

adhesive tape.

7.5. Rotate the device upside down and place the 2nd photomask on top of the glass slide. Expose under UV light at 800 mW of intensity and 8 cm distance for 200 s.

NOTE: See **Figure 1C**. Alignment of the 2nd mask is important.

7.6. Wash the obtained scaffold with DPBS and with cell culture medium that includes 10% fetal bovine serum (FBS).

7.6. Leave them overnight in the 37 °C incubator before seeding the cells.

8. Neonatal rat cardiomyocytes isolation and culture

8.1. Isolate hearts from 2-day-old Sprague-Dawley rats following protocols approved by the Institute's Committee on Animal Care[8].

8.2. Put the heart pieces on the shaker overnight (around 16 h) in 0.05% trypsin without EDTA in HBSS in a cold room.

8.3. Collect the heart pieces with a pipette gun and minimize the amount of trypsin, then put them in a 50 mL tube with 10 mL of warm cardiac media (10% FBS, 1% P/S, 1% L-glutamine).

8.4. Swirl slowly (~60 rpm) in a 37 °C water bath for 7 min. Remove the media carefully from the tube with a 10 mL pipette and leave the heart pieces in the tube.

8.5. Add 7 mL of 0.1% collagenase type 2 in HBSS and swirl in a 37 °C water bath for 10 min.

8.6. Mix with a 10 mL pipette 10x gently to disrupt the heart pieces. Remove the media from the tube with a 1 mL pipette.

8.7. Add 10 mL of 0.1% collagenase type 2 in HBSS and swirl quickly (~120–180 rpm) in a 37 °C water bath for 10 min, then check if the heart pieces are dissolving.

8.8. Mix with a 10 mL pipette, then repeat with a 1 mL pipette to break the last heart pieces.

8.9. Once the solution looks homogeneous, place a 70 µm cell strainer on a new 50 mL tube and pipette the solution 1 mL at a time on strainer.

8.10. Centrifuge the heart cell solution at 180 x *g* for 5 min at 37 °C.

NOTE: If there are still some heart pieces or mucus which did not dissolve, repeat steps 8.7–8.9 again.

8.11. Carefully remove all the liquid above the cell pellet and resuspended the cells in 2 mL of cardiac media.

8.12. Add 2 mL of cardiac media from the tube wall carefully to resuspend the cells and avoid breaking them.

8.13. Add the suspended cells into a T175 flask with warm cardiac media drop by drop. Put the flask in a 37 °C incubator for 1 h to allow cardiac fibroblasts to attach to the bottom.

NOTE: At this preplating step, the cardiac fibroblasts will attach to the flask while the cardiomyocytes will remain in the suspension medium.

8.14. Collect the media from the flask that contains the cardiomyocytes and put it into a 50 mL tube.

8.15. Count the cells, then centrifuge at 260 x *g* for 5 min at 37 °C.

8.16. Resuspend and seed the cells on top of the fabricated soft robot in step 7. Pour 0.4 mL of cardiac media with the cardiomyocytes at a concentration of 1.95×10^6 cell/mL drop by drop onto the entire surface of the device.

8.17. Incubate the samples at 37 °C and change the media with 0.5 mL cell culture media with 2% FBS and 1% L-glutamine on the first and the second days after seeding. Change the media every time the color of the media shifts.

9. Cell staining for alignment analysis

9.1. Remove the media and wash with DPBS for 5 min at RT.

9.2. Fix the cells using 4% paraformaldehyde (PFA) for 20 min at RT. Then wash with DPBS for 5 min at RT.

9.3. Incubate the cells with 0.1% triton in DPBS at RT for 1 h. Wash 3x with PBS for 5 min at RT.

9.4. Incubate the cells with 10% goat serum in DPBS at RT for 1 h.

9.5. Incubate the cells with a primary antibody (sarcomeric α-actinin and connexin-43) in 10% goat serum in DPBS at 4 °C for ~14–16 h.

9.6. Wash 3x with DPBS for 5 min at RT. Incubate the cells with the secondary antibody in 10% goat serum in DPBS at RT for 1 h.

9.7. Wash 3x with DPBS for 5 min at RT, then counterstain cells with 4',6-diamidino-2-phenylindole (DAPI) in DI water (1:1,000) for 10 min at RT. Wash 3x with DPBS for 5 min at RT.

9.8. Take fluorescence images using an inverted laser scanning confocal microscope.

10. Actuator testing and behavior evaluation

10.1. Spontaneous beating of the cardiomyocytes on the soft robot

10.1.1. Incubate bioinspired actuators at 37 °C for 5 days and refresh the media on day 1 and 2 and when necessary (i.e., when the media turns yellow). Use an inverted optical microscope to take images daily (5x and/or 10x). Record cell movements using video capture software on the microscope's live window for 30 s at 20 frames per second (5x and/or 10x) when the contractile activity starts (generally around day 3).

10.1.2. At day 5, detach the membranes by gently lifting from the edge with a blade.

NOTE: If the cells show a strong beating behavior, the membranes will detach by themselves due to the mechanical action of the contractions.

10.2. Bulk electrical signal stimulation

10.2.1. Using a 3 cm spaced PDMS as a holder, affix two carbon rod electrodes with platinum (Pt) wire in a 6 cm Petri dish filled with cardiac media. Then carefully transfer the soft robot into the Petri dish.

10.2.2. Apply a square waveform with 50 ms pulse width, DC offset value 0 V, and peak voltage amplitude between 0.5 and 6 V. The frequency varies between 0.5, 1.0, and 2.0 Hz with a duty cycle between 2.5%, 5%, and 10%, respectively. Record macroscale contractions using a commercially available camera.

10.3. Electrical stimulation with the Au microelectrodes

10.3.1. Attach two copper wires to the Au electrodes though an external square port using silver paste.

10.3.2. Cover the silver paste with a thin layer of PDMS precured at 80 °C for 5 min. Then put the samples on a hot plate at 45 °C for 5 h to fully crosslink the PDMS.

10.3.3. Apply a square wave electrical stimulus on the copper wires with DC offset value 1 V, peak voltage amplitude between 1.5 and 5 V, and frequencies of 0.5, 1.0, and 2.0 Hz respectively.

REPRESENTATIVE RESULTS:

Flow diagram of the steps for developing the Au microelectrode-incorporated bioinspired soft robot

The aim of the soft robot design was to build a membrane capable of actuating a swimming movement with minimal complexity. The structure must be able to sustain strong flexions repeatedly over time (about 1 Hz) and be able to keep its shape while achieving a strong beating. By selectively photo crosslinking the polymer using photomasks, we fabricated a hierarchically structured scaffold comprised of a micropatterned PEGDA hydrogel layer, a flexible Au microelectrodes layer, and a micropatterned CNT–GelMA hydrogel layer. A schematic diagram and actual images of the fabrication procedure of the soft robot as described in the protocol are shown in **Figure 1**. Briefly, there were three main fabrication steps for the bioinspired soft robot with embedded Au microelectrodes: First, a micropatterned PEGDA hydrogel with incorporated Au microelectrodes was obtained by UV crosslinking using the 1st photomask (**Figure 1A**,**B**). Second, a multilayered construct composed of Au microelectrodes, the micropatterned CNT-GelMA, and the PEGDA hydrogels was fabricated by UV crosslinking using the 2nd photomask (**Figure 1C**). Finally, cardiomyocytes were seeded on the fabricated three-layer construct to provide actuation to the soft robot (**Figure 1D**).

Different designs of the soft robot

Regarding the shape of the soft robot, in the beginning, we designed two bioinspired shapes by biomimicking the patterns of two different aquatic animals. The first design was inspired by the appearance of a caraibic starfish (**Figure 2A**, **B**, **C**), because the starfish can be simplified into a two-dimensional (2D) object, has a hard backbone, and has a flexible part that joins together to move in the water, minimizing the required movement. The second device was based on the shape of a manta ray (**Figure 2D**, **E**, **F**) which is easy to reproduce in a 2D device. The manta ray can swim quickly using unique movements. We sketched the manta ray using basic geometric shapes with reduced complexity to be crosslinked during the photomask step. The electrode, placed along the midline of the structure, was designed with a wavy pattern, allowing for a better spread of electrical pulses and flexibility (**Figure 2D**). To develop the bioinspired soft robot, the manta ray-inspired shape was selected and tested thoroughly in this study.

The challenge of embedding the Au microelectrodes between CNT-GelMA and PEGDA hydrogels

The encapsulation of 200 nm thick Au microelectrodes in the fabricated robot body could locally control the construct by providing electrical stimulation. Although the UV crosslinking of both the CNT-GelMA and PEGDA hydrogel patterns directly on the electrode surface hampered the delamination of the electrodes, it guaranteed the successful incorporation of the electrode into the soft robot. However, after transferring the Au electrode on the PEGDA hydrogels, the Au electrode with a rectangular shape and wide width (>1 mm) was easily broken during the fabrication process due to the swelling of the PEGDA hydrogel (**Figure 3A**,**B**,**C**). Hence, we needed to make sure that the microelectrodes were successfully transferred to the PEGDA hydrogel and embedded between the CNT-GelMA and PEGDA hydrogels while intact. Therefore, Au microelectrodes with a serpentine pattern (thickness = 200 μm) were designed and fabricated with soft lithography. Phase contrast microscope pictures with different magnifications and stages were taken in order to inspect signs of fracture on the electrode after transportation on the micropatterned PEGDA hydrogels (**Figure 3D**,**E**,**F**).

The optimization of spacing between hydrogel micropatterns

The cardiomyocyte seeded CNT-GelMA layer showed different beating behavior according to the pattern distances (**Figure 4A**,**B**). This may be attributed to the different ways cells attached to the membrane's surface depending on the lines' distances. In the case of the 50 µm distance, the cells were too packed and did not have the desired organized configuration. The partially interconnected and not aligned cells on the wings were not all simultaneously contributing to the swimming movement. Hence, the force generated by the cardiomyocyte was not enough to bend the wings. At a 150 µm distance, the cells were very well aligned. However, they mainly sat in the groove and there were few interconnections among cells in the upper layers, resulting in weak beating. At a 75 µm distance, the cells were aligned in the bottom part and interconnected in the upper part, showing the strongest beating. In addition, to prevent irreversible complete rolling of the soft robot during the dynamic beating of the cardiomyocytes, we optimized the pattern spacing of the PEGDA hydrogel support layer to 300 µm (**Figure 4C**). Finally, following this parameterization process, we decided to focus more on the manta ray-shaped membrane with 300 µm distance PEGDA patterns and 75 µm distance CNT-GelMA patterns. Cardiac tissue on micropatterned PEGDA- and CNT-GelMA patterns was also shown by phase/contrast images and F-actin/DAPI confocal images (**Figure 4B**).

The analysis of movement of the cardiac tissue on micropatterned PEGDA- and CNT-GelMA hydrogels

To analyze the movement of the actuator, we took videos of the membrane without the Au microelectrodes while applying an electric field using a carbon rod electrode. **Figure 4D** shows some frames taken from the contraction records. It was clearly visible that the manta ray-shaped actuator was bending the wings as expected. The tail was balancing the structure by straightening up a little and the wings were strongly closing in the middle. Some of the membranes showed a rotating movement while contracting due to misaligned micropatterned CNT-GelMA and PEGDA hydrogels (**Figure 4E**). In this case, the movement was less defined compared to the previous one but the contraction was still strong enough to allow actuation of a rotating movement. The total time to complete an entire circle was around 45 s.

The characterization of the cardiomyocytes on the multilayered soft robot and control of beating behavior by electrical stimulation

After seeding and maturation of cardiomyocytes on the bioinspired robotic system (**Figure 5A**), alignment of the cardiac tissue along the direction of the CNT-GelMA patterns was observed (**Figure 5B**-**E**) by both F-actin/DAPI and sacromeric/connexin-43/DAPI immunostaining. Confocal fluorescence images showed well-elongated and aligned cardiomyocytes on the CNT–GelMA hydrogel pattern (**Figure 5B**,**C**). Partial uniaxial sarcomere alignment and interconnected sarcomere structure was observed on the patterned areas (**Figure 5D**). Well-interconnected sarcomere structures of cardiac tissues located directly above the microelectrodes were also observed (**Figure 5E**). To assess the bioinspired soft robot, we detected its function using two methods: First, we applied a biphasic electrical pulse to the soft robot though carbon rod electrodes for artificial tuning and controlling the beating behavior. Second, we connected two copper wires to the outermost end of the Au electrode for generating an electrical signal through the whole robot construct. When we applied an electrical stimulation through the external

carbon electrode or copper wire connected to the Au electrode, the excitation threshold voltage was different at different frequencies (0.5, 1.0, and 2.0 Hz, **Figure 5F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram and actual images depicting the fabrication process of the bioinspired multilayered soft robot electrically controlled by electrical signal via the integration of flexible Au microelectrodes. (**A**) Patterning and crosslinking of the PEGDA hydrogel using the 1st photomask. (**B**) Micropatterned PEGDA hydrogel with the encapsulated Au microelectrodes on the TMSPMA glass obtained after step (**A**). (**C**) Crosslinking of the CNT-GelMA patterned hydrogel using the 2nd photomask. (**D**) Seeding of the cardiomyocytes on the multilayered construct.

Figure 2: Design of the bioinspired soft robots. (**A**) Real starfish picture and different views of the three-dimensional (3D) CAD model pointing out the components and stripes. (**B**) Mask design for CNT-GelMA pattern, PEGDA pattern, and Au microelectrodes for the starfish shape. (**C**) Optical microscope image of the micropatterned CNT-GelMA and PEGDA patterns for the starfish shape. (**D**) Real manta ray picture and different views of the 3D CAD model pointing out the components. (**E**) Mask design for CNT-GelMA pattern, PEGDA pattern, and Au microelectrodes for the manta ray shape, adapted with permission from Su Ryon et al.[10]. (**F**) Optical microscope image of the micropatterned CNT-GelMA and PEGDA patterns for the manta ray shape.

Figure 3: Design of the flexible Au microelectrodes. (**A**) Photograph of fabricated Au electrodes with rectangular shapes and wide widths. (**B** and **C**) Optical microscope images of Au electrodes that failed to transfer to the PEGDA hydrogels. (**D**) Wavy Au microelectrodes before and after (**E** and **F**) being transferred on the micropatterned PEGDA hydrogel.

Figure 4: The optimization of micropatterned PEGDA and CNT–GelMA hydrogels and movement analysis of soft robots. (**A**) Optical images of cardiomyocytes on the CNT–GelMA hydrogel pattern with 50, 75, and 150 μm spacing. (**B**) Optical images and F-actin/DAPI staining of cardiomyocytes on the PEGDA- and CNT-GelMA hydrogel patterns with 300 μm and 75 μm spacing, respectively. (**C**) The rolling morphologies of the bioinspired constructs with and without the micropatterned PEGDA hydrogel with 300 μm spacing. (**D**) Frames of the free-standing bioinspired soft robot video recorded while applying the electric stimulus. (**E**) Collage of four different frames taken from the video recording the rotating movement of the soft robot.

Figure 5: Characterization of cardiomyocytes on Au microelectrode-incorporated soft robot and control of beating behavior by electrical stimulation. (**A**) Optical microscope image of the cultured cardiomyocytes on the Au microelectrodes encapsulated between PEGDA and CNT-GelMA hydrogels. (**B**) F-actin/DAPI fluorescence image showing the well-elongated and aligned cardiomyocytes on the CNT–GelMA hydrogel micropattern. (**C**-**E**) Confocal fluorescence images showing sarcomere alignment and interconnected sarcomere structures on the fabricated soft robot: (**C** and **D**) cultured cardiomyocytes on the CNT–GelMA hydrogel micropattern, and (**E**) near the Au microelectrodes. (**F**) Required excitation threshold voltage at different frequencies (0.5,

1.0, and 2.0 Hz) when applying electrical stimulation via carbon rod electrode and embedded Au microelectrodes.

DISCUSSION:

Using this method, we were able to successfully fabricate a batoid fish-like bioinspired soft robot with an integrated self-actuating cardiac tissue on a multilayer structured scaffold that is controlled by embedded Au microelectrodes. Due to two distinct micropatterned hydrogel layers made of PEGDA and CNT–GelMA hydrogels, the bioinspired scaffold showed good mechanical stability and ideal cell alignment and maturation. The PEGDA pattern layer, which serves as a cartilage joint of the skeletal architecture in a sting ray, provides mechanical support for the whole robot body. Specifically, it maintained mechanical stability during cardiac tissue contraction and relaxation, while allowing for efficient beating due to its ability to release the membrane tension following contraction. Furthermore, the nanometric thickness of the microelectrodes (200 nm), as well as their serpentine pattern, allowed them to be flexible enough to not impede or influence the contraction of the cardiac tissue (**Figure 2**). To easily transfer microelectrodes on the hydrogel surface without any breakage, Au microelectrodes were fabricated on the glass without any adhesion layer, such as titanium, which is commonly used to create strong adhesion between the glass and Au. Meanwhile, the CNT-GelMA layer, which provides support for cardiomyocyte attachment and alignment, was made with patterns perpendicular to the orientation of the PEGDA hydrogel pattern (**Figure 3**). After maturation, the cardiomyocytes on the top layer provided self-actuation for the whole scaffold. Through the local electrical stimulation of the incorporated Au flexible microelectrodes, we could modulate the beating frequency of the robot without harming the cardiac tissue on it. Although this fabrication method is easy to learn and to reproduce, there are still a few technically challenging steps in the fabrication process that need to be emphasized.

There are five critical steps for the fabrication of the soft biorobot: 1) correct dispersion of the CNTs in the GelMA hydrogel; 2) successful UV crosslinking of the PEGDA and CNT-GelMA hydrogels on the TMSPMA-coated glass; 3) transfer of the Au microelectrodes from the support glass to the hydrogel pattern; 4) correct detachment of the actuator from the supporting glass slide; 5) creation of good electrical contact between the Au microelectrodes and the wires used for the connection to the waveform generator.

Compared with pristine GelMA substrates, the incorporation of CNTs provides the GelMA hydrogel with enhanced mechanical properties and advanced electrophysiological functions that contribute to higher spontaneous synchronous beating rates and a lower excitation threshold of myocardial tissue[9]. The problem of CNT cytotoxicity is prevented not only by using surface functionalized CNTs but also by incorporating the nanostructures in the GelMA hydrogel matrix up to a concentration of 5.0 mg/mL[9]. In fact, the interaction between the hydrophobic segments of the GelMA hydrogel with the CNTs sidewalls lead to the encapsulation of CNTs in the hydrogel porous matrix[14]. This not only prevents them from forming potentially toxic aggregates, but it also enhances CNTs solubility in saline solutions (e.g., DPBS or cell culture medium).

To successfully incorporate the Au microelectrodes between the PEGDA and CNT-GelMA

hydrogels, specific attention needs to be put into the UV crosslinking of each single layer. Specifically, to transfer the Au microelectrodes on the PEGDA hydrogel layer, it is necessary to ensure that the hydrogel solution covers the entire electrode area to avoid the rupture of the electrodes during the peeling step. Therefore, the quality of the TMSPMA glass coating is fundamental to guarantee an optimal adhesion of the PEGDA hydrogel onto the glass substrate, thereby preventing its detachment during the transfer step of the microelectrodes.

Another critical step of the method is the detachment of the bioactuator from the supporting glass slide. This problem can be easily solved when the spontaneous beating of the cardiac tissues is synchronous and strong enough to naturally peel the supporting hydrogel from the glass slide. For this reason, as reported before, it is fundamental to optimize the hydrogel patterns to induce a specific cell alignment favorable for the organization of a functional and synchronous cardiac tissue.

To electrically connect the microelectrodes to the waveform generator, electrical connections must be created on the microelectrodes. During this step, it is important to completely encapsulate the silver glue used for contacting the microelectrodes to the copper wire to avoid cytotoxic effects. This is successfully achieved by depositing a thin drop of PDMS on the top of the electrical contact.

This method could not only overcome the limitations of existing optogenetic techniques, such as complicated fabrication processes, long fabrication times and potential toxicity of optogenetic tools, but also strongly enhance the performance of cell-based actuators leading to real-time stimulation using low-cost and easy-to-handle techniques. Although the design of our current bioinspired actuators could not generate forward propulsion, its success in the field of autonomous cell-based robots could attract a lot of interest. This method can also potentially contribute to the development of wirelessly-powered implantable patches for a whole robot body. This method paves the way for future wireless electrical stimulation of soft-biorobots though the integration of flexible RF circuits directly in the hydrogel-based scaffold.

ACKNOWLEDGMENTS:

This paper was funded by the National Institutes of Health (R01AR074234, R21EB026824, R01 AR073822-01), the Brigham Research Institute Stepping Strong Innovator Award, and AHA Innovative Project Award (19IPLOI34660079).

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

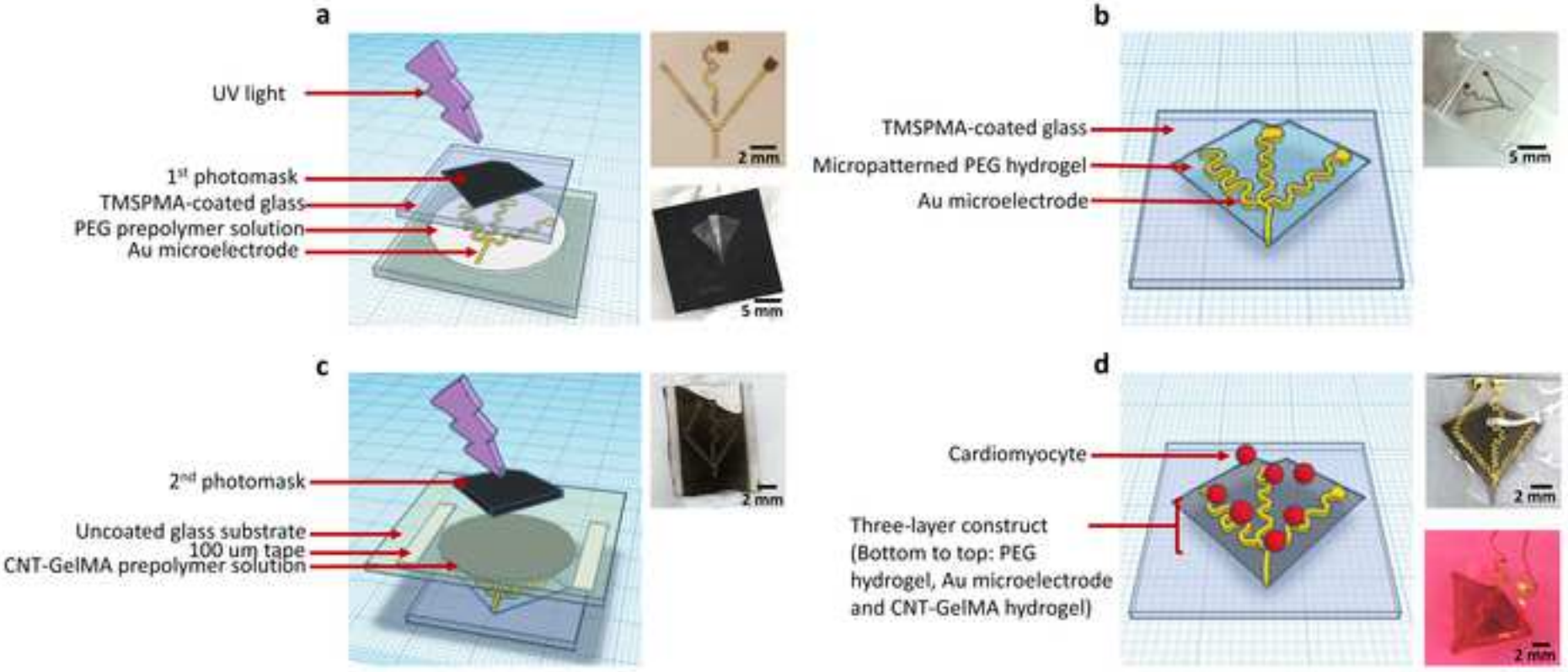

Figure 2

Click here to access/download;Figure;figure 2.TIF

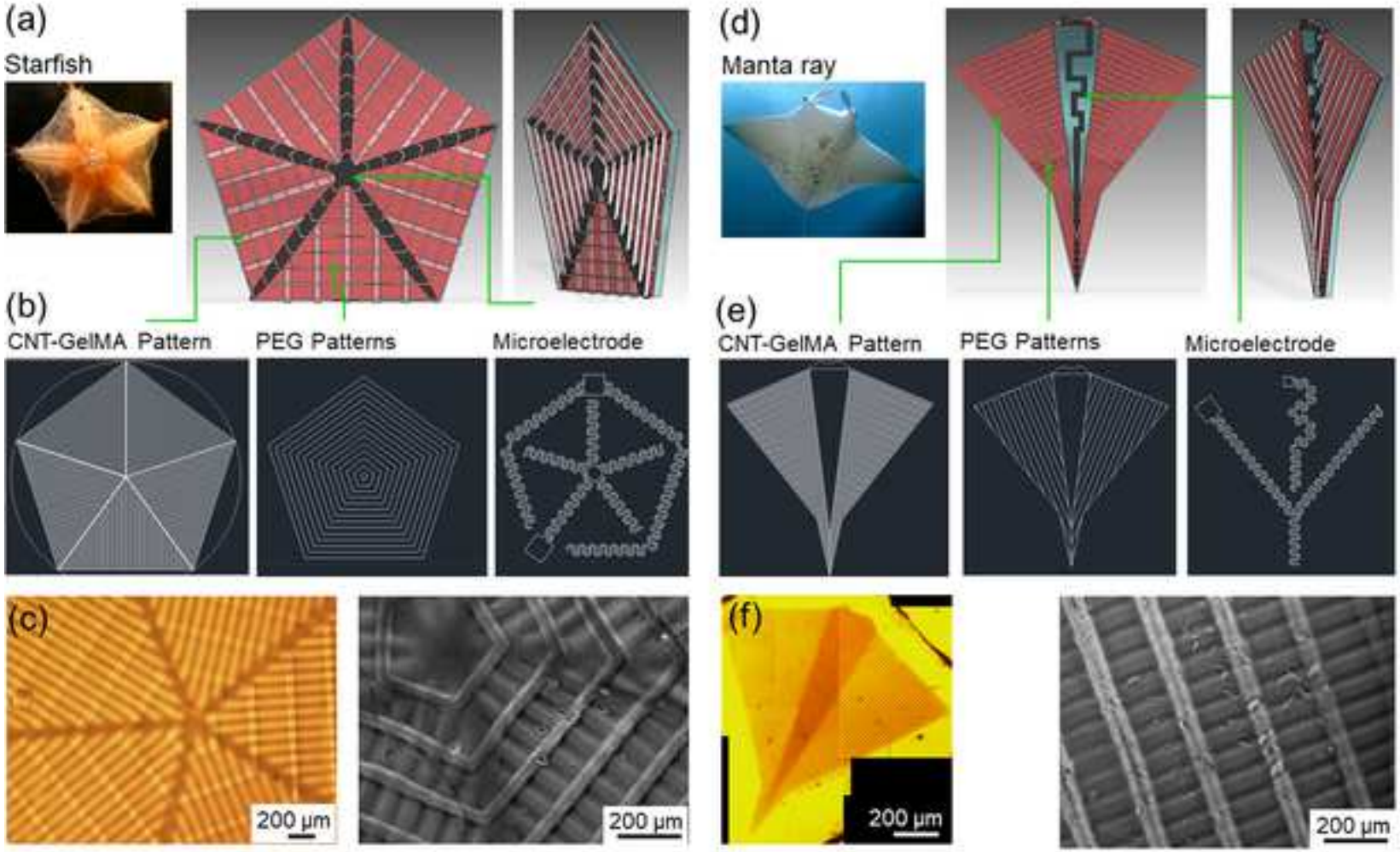

Figure 3

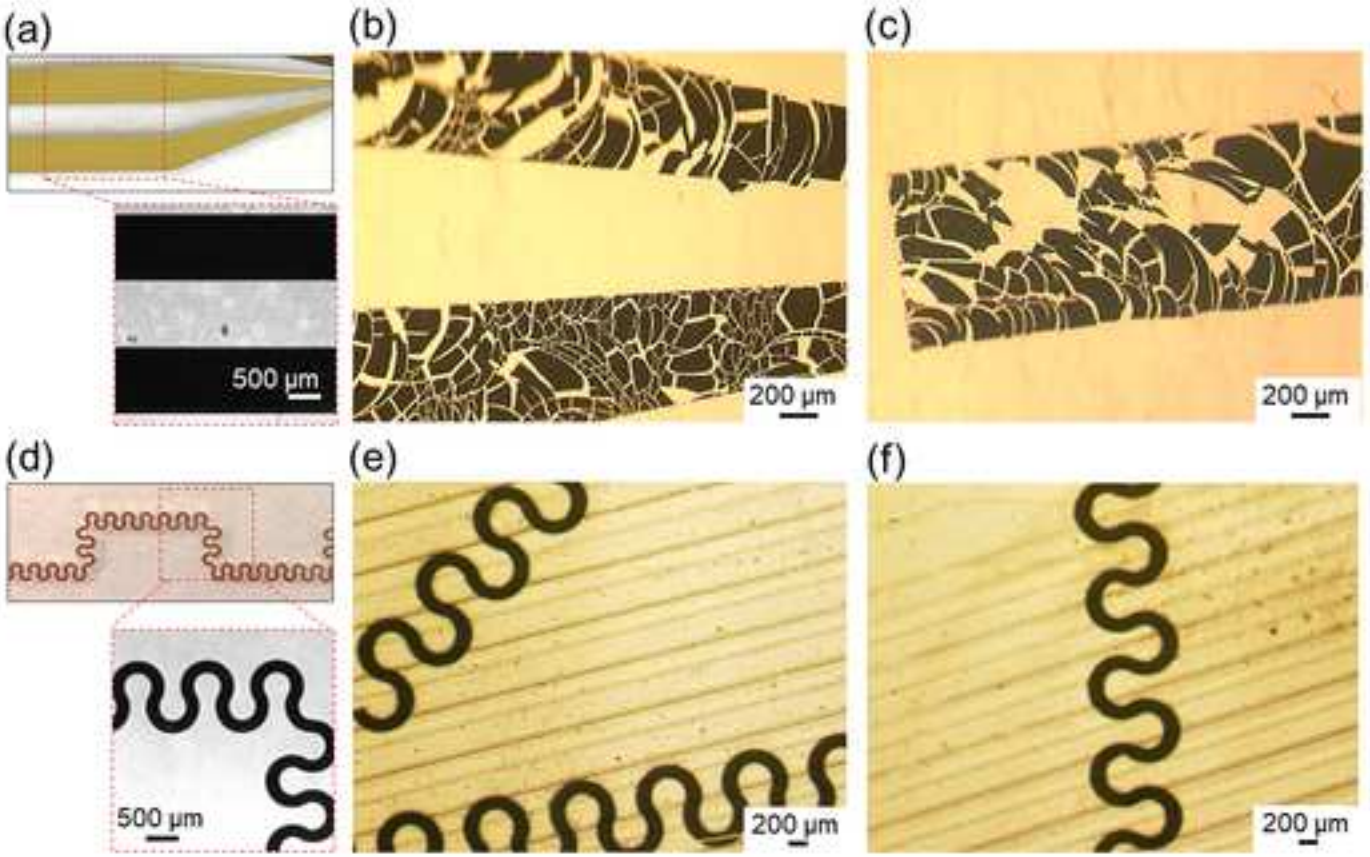

Figure 4

Click here to access/download;Figure;figure 4.tif

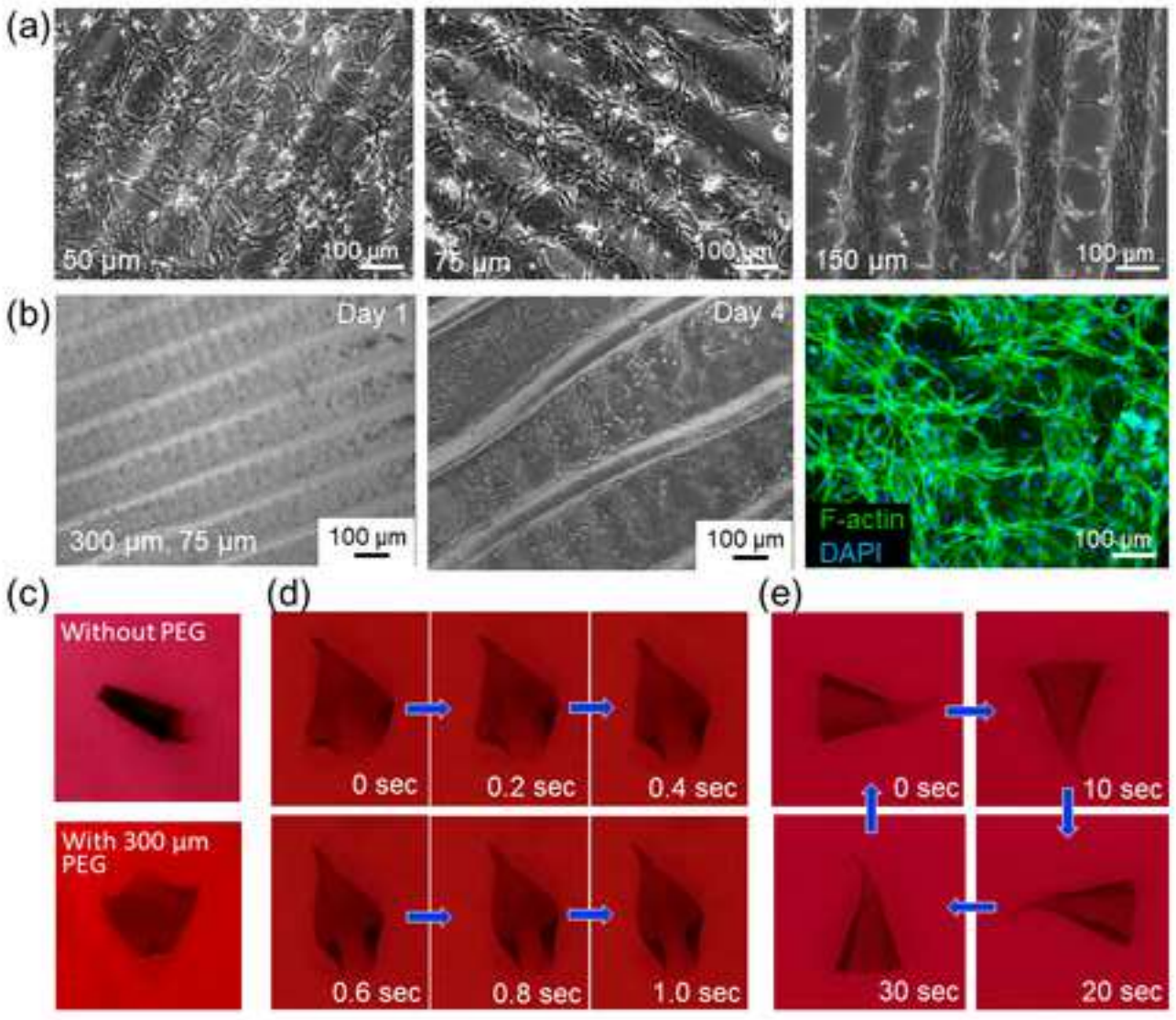

Figure 5

Click here to access/download;Figure;figure 5.tif

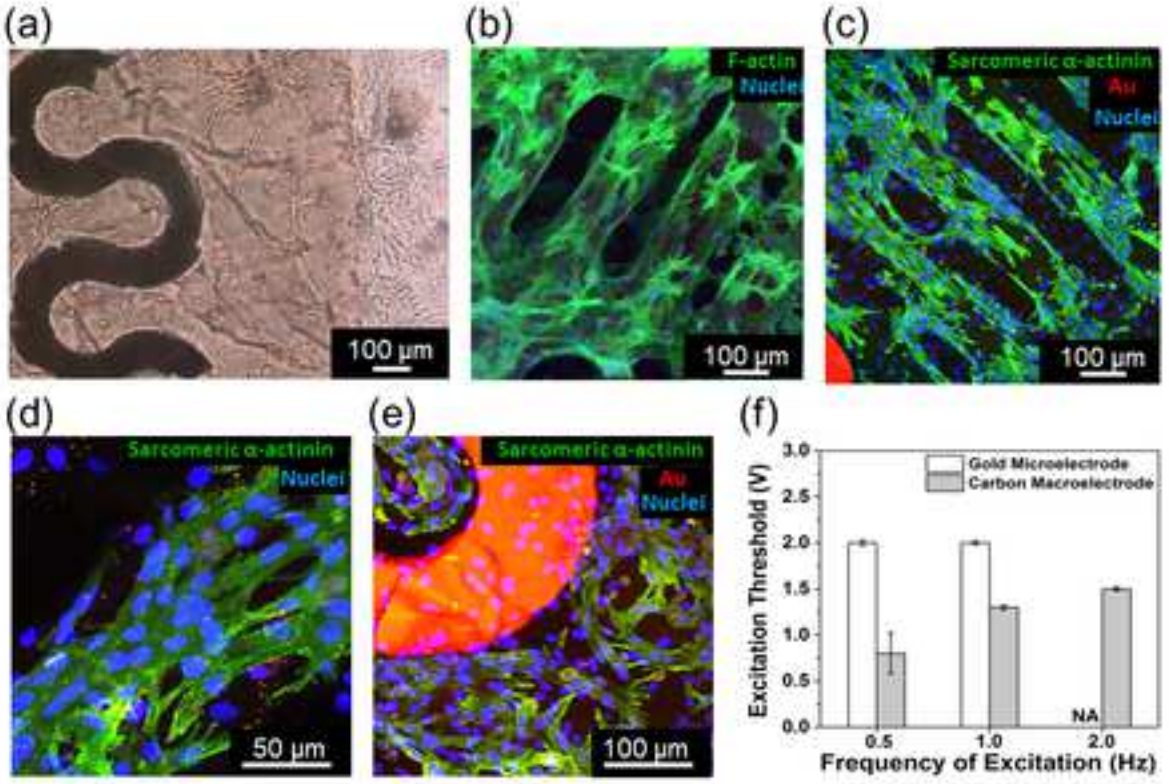

Table of Materials

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
250 mL Beaker	PYREX	1000-250CNEa	
2-Hydroxy-4′ -(2-hydroxyethoxy)-2-methylpropiophenone	Sigma-Aldrich	410896	
3-(Trimethoxysilyl)propyl methacrylate	Milipore	M6514	
37° Water bath	VWR	W6M	
4’,6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich	D9542	
50mL Conical Centrifuge Tubes	Falcon	14-959-49A	
70 µm Cell Strainer	Falcon	352350	
80° incubator	VWR	1370GM	
Alexa Fluor 488 goat anti-mouse IgG (H+L)	Invitrogen	A11029	
Alexa Fluor 594 goat anti-rabbit IgG (H+L)	Invitrogen	A11037	
Alexa Fluor 488 Phalloidin	Invitrogen	A12379	
Antibiotic/Antimycotic solution	ThermoFisher Scientific	15240062	
Anti-Connexin 43/GJAI antibody	Abcam	ab11370	Rabbit polyclonal
Anti-Sarcomeric α-actinin	Abcam	ab9465	Mouse monoclonal
Benchtop Freeze Dryers	Labconco	77500-00 K	
Biosafety cabinet	Sterilgard	A/B3	
Carbon rod electrodes	SGL Carbon Group	6971105	
Centrifuge	Eppendorf	5804	
CO_2 incubator	Forma Scientific	3110	
Collagenase, Type II, Powder	Gibco	17-101-015	
Confocal Microscope	Zeiss	LSM 880	
COOH Functionalized Carbon Nanotubes	NaonoLab	PD30L5-20-COOH	
Dicing saw machine	Giorgio Technology	DAD-321	
DMEM, High Glucose	Gibco	11-965-118	
DPBS without Calcium and Magnesium	Gibco	14-190-144	
E-beam evaporator	CHA	57367	
Fetal Bovine Serum	Gibco	10-437-028	
Gelatin	Sigma-Aldrich	G9391	Type B, 300 bloom from porcine skin
Glass slide	VWR	48382-180	
HBSS without Calcium, Magnesium or Phenol Red	Gibco	14-175-079	
Inverted optical microscope	Olympus	CK40	
Magnetic hotplate	Corning	PC-420	
methacrylic anhydride	Sigma-Aldrich	276695	Contains 2,000 ppm topanol A as inhibitor
Nunc EasYFlask 175cm^2	ThermoFisher Scientific	159910	
Olicscope	Siglent	SDS1052DL+	
Paraformaldehyde Aqueous Solution -16%	Electron Microscopy Sciences	15710	
PDMS SYLGARD 184	Sigma-Aldrich	761036	
Photomask	Mini micro stencil inc		
Platinum wire	Alfa Aesar	AA43014BU	
Polyethylene glycol dimethcrylate	Polysciences Inc.	15178-100	
Regenerated Cellulose Dialysis Tubing	Fisherbrand	21-152-14	
Silver Epoxy Adhesive	MG Chemicals	8330S	
Stericup Quick Release-GP Sterile Vacuum Filtration System	Millipore	S2GPU02RE	
Ultra sonicator	Qsonica	Q500	
UV Curing System	OmniCure	S2000	
Vortex mixer	Scientific Industry	SI-0246A	
Waveform generator	Agilent	33500B	
Wrap Aluminium foil	Reynolds	N/A	

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October 18, 2019

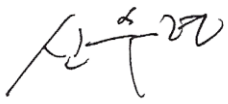
Subject: Revised manuscript based on reviewers' comments

Dear Alisha DSouza,

We would like to thank you and the reviewers for the comments and the enthusiasm for the submitted manuscript. Please find the attached response letter including the specific comments from the reviewers and yourself along with our explanation and corrected statements. All the changes are tracked within the revised version of the manuscript to show all the edits. We believe that after the revisions made while to addressing the comments of the reviewers, our manuscript has been significantly improved in quality and scope. We hope you will find the manuscript suitable for publication in *Jove*.

We appreciate your consideration for the publication of this work. If you have any questions, please feel free to contact me at sshin4@bwh.harvard.edu or (617) 835-1164.

Respectfully yours,



Su Ryon Shin, Ph.D.

Response to the referees' comments

We thank the editors and reviewers for their time and their valuable feedback on our manuscript. We have revised the manuscript in accordance with their suggestions. Please find the reviewers' comments in blue and our response in black.

Editorial Comments:

• Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write lines … avoid this overlap.

Answer: We have checked duplication of our manuscript using an ithentication program. The similarity index is around 6%. We couldn't find any similar sentences in the results. We have attached the ithentication result.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

Answer: We have added more specific details to our protocol steps. We hope the protocol contain enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. The modified parts were highlighted with pink color.

1) 3.2: Mention frequency (Hz) and amplitude (in Watt): 0.66Hz and 100 Watt were added.

2) 6.1, 7.1: provide the CAD file as a supplementary file. What are the specifications? We provided three CAD files.

3) 6.5: How is the Au layer deposited? Mention all settings: Au layer was deposited by using E-bean evaporator (Denton EE-4, Vacuum (Torr): 10^{-6}, Power (%): 2.6, Rate (Å/sec): 2)

4) 7.6: mention temperature: The temperature as 37 ^{o}C was added.

5) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.: We have added the ethics statement on pages 5 and 6.

"This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the institutional Animal Care and Use Committee (IACUC) of the Brigham and Women's Hospital."

6) 10.1.1: mention magnification and other settings. We have added the magnification in the section.

• Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to

identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Answer: We have highlighted the steps with yellow color that should be visualized.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Answer: We have modified the content of discussion to meet the requirements of Jove that were highlighted with green color on page 13.

"This method could not only overcome the limitations of existing optogenetic techniques, such as complicated fabrication processes, long fabrication times and potential toxicity of optogenetic tools, but also strongly enhance the performances of cell-based actuators leading to real-time stimulation using low-cost and easy-to handle techniques. Although the design of our current bioinspired actuators could not generate forward propulsion, its encouragement in the field of autonomous cell-based robots could attract a lot of interest."

• Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are MINI MICRO STENCIL INC. (USA)., CAD/Art Services, Inc, falcon tube, etc.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Answer: We have replaced all commercial sounding language in our manuscript with generic names that were highlighted with sky color.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher

(this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation].

Answer: We reused figure 2e and figure 5f from our previous publication. We have obtained explicit permission to reuse the figures from the previous publisher. We have provided the permission on supplementary documents and we have also cited the figures appropriately in the figure legend.

Comments of Reviewers

We would like to thank the reviewers for their comments. As follows, we will answer each question about the experimental protocol one by one.

Reviewers #1:

1. 6.4: How is the tape placed on the glass substrates exactly?

Response: The position of double sided tape does not matter as long as the tape does not block the space for the micropatterned Au electrode. The two pieces of tape were placed manually on the support at a distance short enough to host the glass, and large enough to fit the entire pattern. We also modified the content in 6.4 to provide readers with a more precise description. (page: 4: highlighted in green).

"The two pieces of tape were placed manually on the support at a distance short enough to host the glass, and large enough to fit the entire pattern."

2. 6.5: What sizes are the microelectrodes cut to?

Response: The size of cut electrodes is 7.376mm x 8.9mm x 200nm, which was added in 6.5. (page: 5: highlighted in purple).

3. 7.2: What is used to cover the glass slides? There seems to be more than 1 task in this step and unclear is the reference to glass slide is the same as the TMSPMA coated glass slide.

Response: We apologize for the confusing description of the fabrication process. Here, we will explain the procedure more in detail and will provide a schematic diagram to show the process more clearly. First, we added 15μL of PEGDA pre-polymer solution on top of the Au microelectrode. Second, we put a TMSPMA coated glass slide above the PEGDA pre-polymer solution. Third, we put 1st photomask (micropatterned PEGDA) above the TMSPMA coated glass slide. Finally, we exposed whole construct to UV light to obtain the micropatterned PEG hydrogel with the Au microelectrode. We have modified the procedure in 7.2 and modified the schematic diagram in Figure 1. (pages 5 and 11: highlighted with grey color).

"Pour 15 μL of 20% PEGDMA pre-polymer solution on top of the Au microelectrode, then cover with the TMSPMA coated glass slide. Place the 1st photomask for the glass slide (micropatterned PEGDA) on top of the TMSPMA coated glass slide and expose whole construct to UV light (200-Watt mercury vapor short arc lamp with 320-390 nm filter) at 800 mW of intensity and 8 cm distance for 110 s."

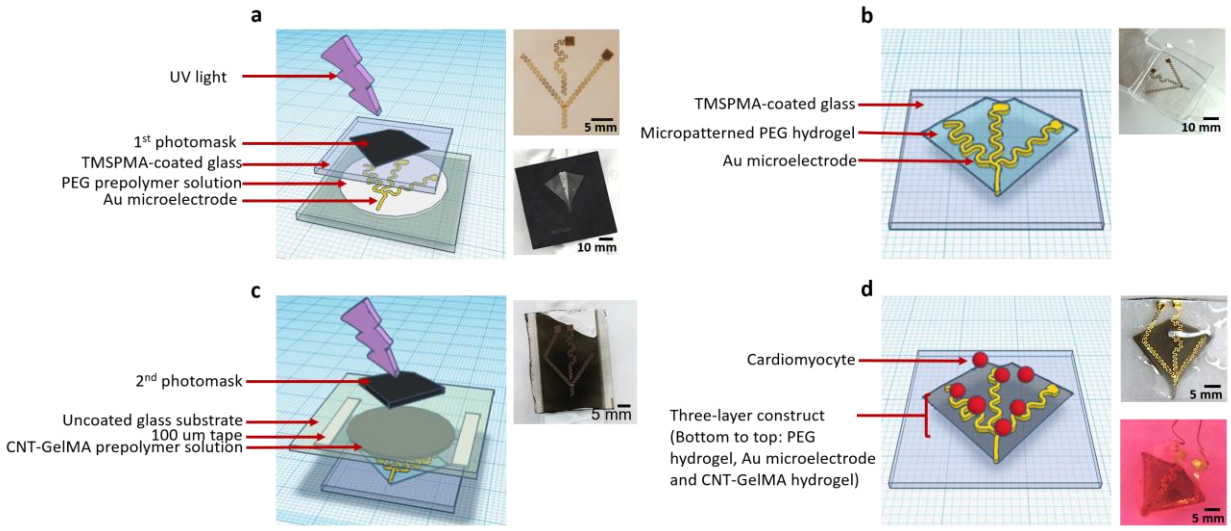


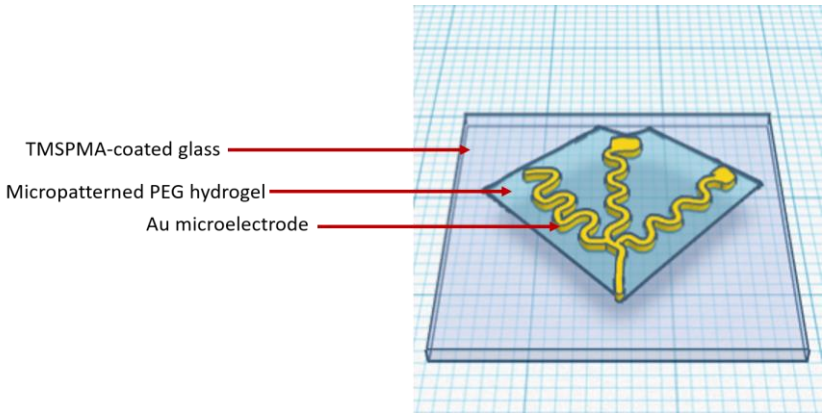
Figure 1: Schematic diagram and actual images depicting the fabrication process of the bio-inspired multilayered soft robot which is electrically controlled by electrical signal via the integration of a flexible Au microelectrode. *(a) Patterning and crosslinking of the PEGDA hydrogel using the 1st photomask. (b) Micropatterned PEGDA hydrogel with encapsulated Au microelectrode on the TMSPMA glass obtained after step (a). (c) Crosslinking of the CNT-GelMA patterned hydrogel using the 2nd photomask. (d) Seeding of the cardiomyocytes on the multi-layered construct.*

4. 7.3: Need to specify what is being detached and from what?

Response: During crosslinking, we micropatterned the PEGDA hydrogel by using a photomask on top of the Au microelectrode substrate. A TMSPMA-coated glass slide was placed between the photomask and the PEGDA hydrogel to control the final thickness thanks to the tape spacers. Then, we detached the *micropatterned PEGDA hydrogel together with the Au microelectrode* from the *uncoated glass substrate* (shown in the above schematic diagram). Due to the TMSPMA coating, the construct was transferred from the uncoated glass substrate to the TMSPMA-coated one. Hence, after this step, we obtained a TMSPMA coated glass slide on which the micropatterned PEGDA hydrogel and the Au microelectrode were placed (shown in the below schematic diagram). We added the explanation of the procedure in 7.3 and modified the schematic diagram in Figure 1. (page 5: highlighted with green color; page 11: heighted with grey color).

"Add DPBS to surround the glass slide and detach the micropatterned PEGDA hydrogel together with the Au microelectrode from the uncoated glass substrate carefully after 5 to 10 min to obtain the glass slide that has the micropatterned PEGDA hydrogel with the Au microelectrode.

Note: Look at Figure 1b. Due to the TMSPMA coating, the construct is transferred from the uncoated glass substrate to the TMSPMA-coated one…"



Reviewer #2:

1. The clear description of the figures and caption. More detailed sub-caption should be added. it is good for readers to follow the work. The protocol should be well connected with the equipment in text

Response: We thank the reviewer for pointing this out. We have modified the content of *Representative results* (pages: 8, 9 and 10: highlighted in grey) and added sub-captions to provide clear descriptions of the figures (pages: 8, 9 and 10: highlighted in grey).) We also modified the *schematic diagram* (Figure 1) of the fabrication process to make this protocol easy to understand. In addition, we modified the equipment text in *Table of Material* to make this protocol more reproducible.

"…. Briefly, there are three main fabrication steps for the bioinspired soft robot with an embedded Au microelectrode. First, a micropatterned PEGDA hydrogel with an incorporated Au microelectrode was obtained by UV crosslinking using the 1st photomask (Figure 1a and b). Second, a multilayered construct composed of the Au microelectrode, the micropatterned CNT-GelMA and the PEGDA hydrogels was fabricated by UV crosslinking using the 2nd photomask (Figure 1c). Finally, cardiomyocytes were seeded on the fabricated three-layer construct to provide actuation to the soft robot (Figure 1d)."

*"**1. Flow diagram schematizing the main steps for developing the Au microelectrode incorporated bioinspired soft robot**….*

2. Different designs of the soft robot*….*

3. The challenge of embedding the Au microelectrode between CNT-GelMA and PEGDA hydrogels*….*

4. The optimization of spacing between hydrogel micropatterns*….*

5. The analysis of movement of the cardiac tissue on micropatterned PEGDA- and CNT-GelMA hydrogels*….*

6. The characterization of the cardiomyocytes on the multi-layered soft robot and control of beating behavior by electrical stimulation*…."*

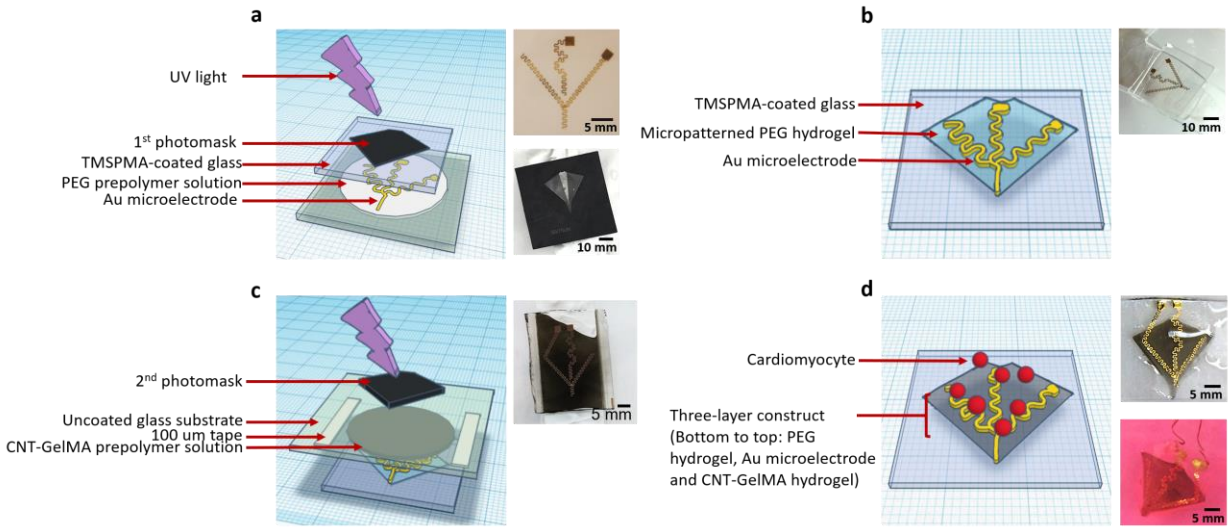


Figure 1: Schematic diagram and actual images depicting the fabrication process of the bio-inspired multilayered soft robot which is electrically controlled by electrical signal via the integration of a flexible Au microelectrode. *(a) Patterning and crosslinking of the PEGDA hydrogel using the 1st photomask. (b) Micropatterned PEGDA hydrogel with encapsulated Au microelectrode on the TMSPMA glass obtained after step (a). (c) Crosslinking of the CNT-GelMA patterned hydrogel using the 2nd photomask. (d) Seeding of the cardiomyocytes on the multi-layered construct.*

Reviewer #3:

1. The article needs of a flow diagram schematizing the main steps for developing the soft robot. The current images, contained in the Figures, presented by the authors are not quite effective in depicting the fabrication of the device

Response: We thank the reviewer for pointing this out. We have modified the content of *Representative results* (pages: 8, 9 and 10: highlighted in grey) and added sub-captions, as well as the *schematic diagram* of the fabrication process (Figure 1) to provide the readers with a clearer description of the fabrication procedure. We hope that our protocol, together with the new flow diagram, will improve the readers' understanding of the protocol.

*"**1. Flow diagram schematizing the main steps for developing the Au microelectrode incorporated bioinspired soft robot***

.... Briefly, there are three main fabrication steps for the bioinspired soft robot with an embedded Au microelectrode. First, a micropatterned PEGDA hydrogel with an incorporated Au microelectrode was obtained by UV crosslinking using the 1st photomask (Figure 1a and b). Second, a multilayered construct composed of the Au microelectrode, the micropatterned CNT-GelMA and the PEGDA hydrogels was fabricated by UV crosslinking using the 2nd photomask (Figure 1c). Finally, cardiomyocytes were seeded on the fabricated three-layer construct to provide actuation to the soft robot (Figure 1d)."

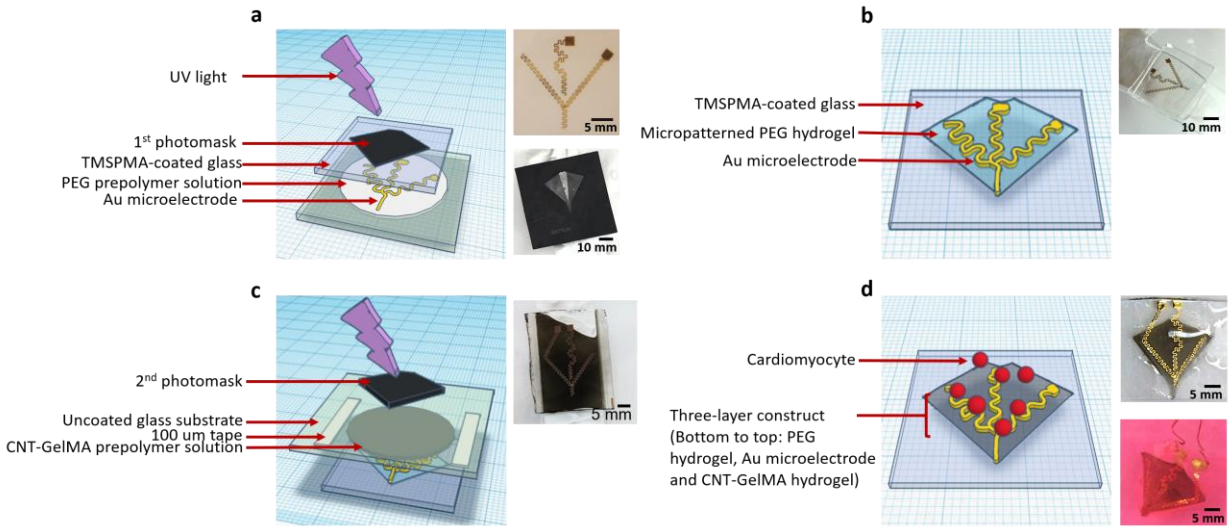


Figure 1: Schematic diagram and actual images depicting the fabrication process of the bio-inspired multilayered soft robot which is electrically controlled by electrical signal via the integration of a flexible Au microelectrode. *(a) Patterning and crosslinking of the PEGDA hydrogel using the 1st photomask. (b) Micropatterned PEGDA hydrogel with encapsulated Au microelectrode on the TMSPMA glass obtained after step (a). (c) Crosslinking of the CNT-GelMA patterned hydrogel using the 2nd photomask. (d) Seeding of the cardiomyocytes on the multi-layered construct.*

2. The authors should make available the CAD blueprints/renders, as with no access to those files it will be practically impossible to reproduce the robots.

Response: Thank you very much for your comment. We have provided our CAD files of the Au microelectrode, the PEGDA micropattern mask and the CNT-GelMA micropattern mask as supplementary documents.

3. Images depicting the actual setup used for fabricating the device are required. Particularly for the "photo-curing" part of the work.

Response: Thank you very much for your suggestion. Based on your comment, we modified the images depicting the actual setup (Figure 1) and added a detailed description of these images in *Representative results* to show the fabrication process more clearly. We added the explanation of the procedure on page 9 and new schematic diagram in Figure 1. (page: 9: highlighted in grey).

"…. Briefly, there are three main fabrication steps for the bioinspired soft robot with an embedded Au microelectrode. First, a micropatterned PEGDA hydrogel with an incorporated Au microelectrode was obtained by UV crosslinking using the 1st photomask (Figure 1a and b). Second, a multilayered construct composed of the Au microelectrode, the micropatterned CNT-GelMA and the PEGDA hydrogels was fabricated by UV crosslinking using the 2nd photomask (Figure 1c). Finally, cardiomyocytes were seeded on the fabricated three-layer construct to provide actuation to the soft robot (Figure 1d)."

4. Clarify what type of UV-lamp used and the reactor/irradiation system they used.

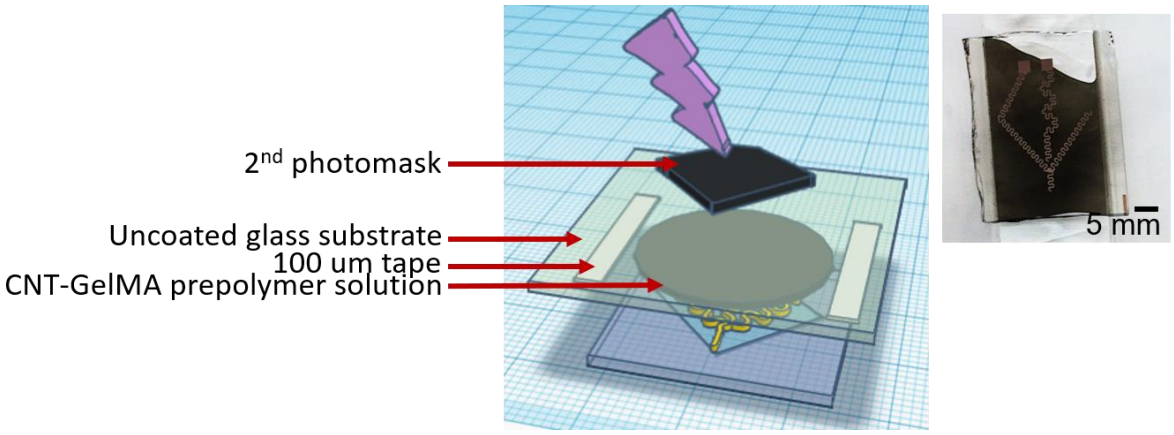
Response: A 200-Watt mercury vapor short arc lamp was used. A mercury-vapor lamp is a gas discharge lamp that uses an electric arc through vaporized mercury to produce light. When electricity is applied to the lamp, this mercury is "excited" and emits UV light. The exact wavelengths emitted depend on the vacuum pressure within the lamp tube itself. Here, the lamp uses high pressure to obtain 320-390nm UV light with filters (OmniCure, S2000). We added the explanation of the UV-lamp on page 5. (page: 5: highlighted in grey).

"…Place the 1st photomask the glass slide (micropatterned PEGDA) on top of the TMSPMA coated glass slide and exposed whole construct to UV light (200-Watt mercury vapor short arc lamp with 320-390 nm filter) at 800 mW of intensity and 8 cm distance for 110 s."

5. Point 7.4 is confusedly written, please revise the verbal tenses and reduce length. Again, a schematic diagram depicting the main/key steps will be of great help in this section.

Response: Thank you very much for your idea. We have revised the content. We hope the modified content and the new schematic diagram will be helpful for readers to understand and to reproduce the soft robot. Here, the modified protocol and corresponding schematic diagram are attached (Figure 1c) to provide additional information. We modified the explanation of the procedure on page 5 and the schematic diagram in Figure 1. (page: 5: highlighted in dark yellow).

"7.4 Place 100 µm spacers made by stacking two layers of commercial invisible tape (thickness: 50 µm) on the bottom of a petri dish. Deposit a drop of 20 µL CNT-GelMA pre-polymer solution between the spacers and then flip the glass slide obtained in 7.3 and fix it onto the dish with adhesive tape. Then, rotate the device upside-down and place the 2nd photomask on the top of the glass slide, and expose under UV light at 800 mW of intensity and 8 cm distance for 200 s."



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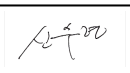
Signature:	
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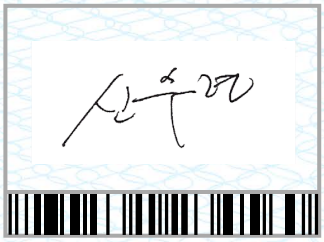

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