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3D Fabrication of Biological Machines

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***Abstract*— Cell-based biological machines can be defined as a set of sub-components consisting of living cells and cell-instructive micro-environments that interact to perform a range of prescribed tasks. The realization of biological machines and their sub-components will require a number of suitable cell sources, biomaterials, and enabling technologies. Here, we review our group's recent accomplishments and continuing efforts toward the development of building biological machines.**

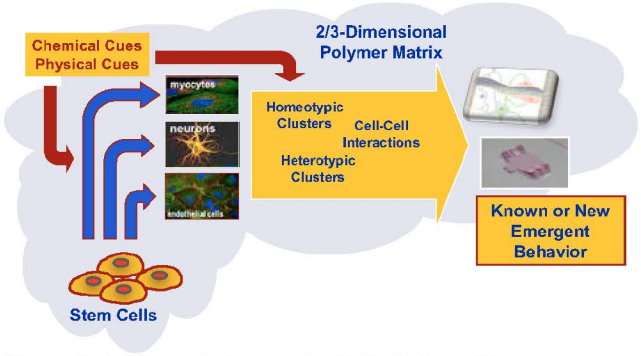

Figure 1. A schematic image of a biological machine.

I. Introduction

What are biological machines? Much like conventional machines made out of hard materials, biological machines (or 'bio-machines') are cellular systems that can be programmed to perform prescribed tasks (**Figure 1**). The difference is that these machines are made out of soft materials and include biological entities, such as DNA, proteins, or living cells. Each functional component of a bio-machine serves as a building block having a particular function, such as sensing, information processing, actuation, protein expression, and transport.[1] These building blocks and their corresponding functionalities can be combined to create complex bio-machines that include but are not limited to applications in health, security, and the environment. One high-level example is a bio-machine that can sense a toxin in the external environment, move towards the source of that toxin, and deliver drugs to neutralize it. As part of an ever increasing discipline, we seek to develop new and more complex biological machines.

How do we propose to build a biological machine? First, we choose a cell source. All mammalian cells, for example, originate from stem cells and progenitor cells that differentiate into a diverse cluster of specialized cell types. These specialized cell types not only differ in their appearance, but also in their function. For example, neurons can sense and process information, muscle cells can actuate, and endothelial cells can transport. Second, we select suitable biomaterials that support the cells of choice. These biomaterials serve as scaffolding that keep the cells alive and provide instructive cues, both physical and chemical, that help cells perform their designated functions. Hydrogels are a class of biomaterials that can be designed for this purpose. Third, we utilize a three-dimensional (3D) printer that assembles the cells and biomaterials into functional components that form the building blocks of a bio-machine. The 3D printer can print a cluster of muscle cells and its muscle-specific micro environment and combine it with a cluster of neurons and its neuron-specific micro environment. These building blocks can then integrate, coordinate, and create new functionalities that determine the bio-machine's tasks. Here, we review our group's past accomplishments and outline our recent efforts toward the development of more complex bio-machines.

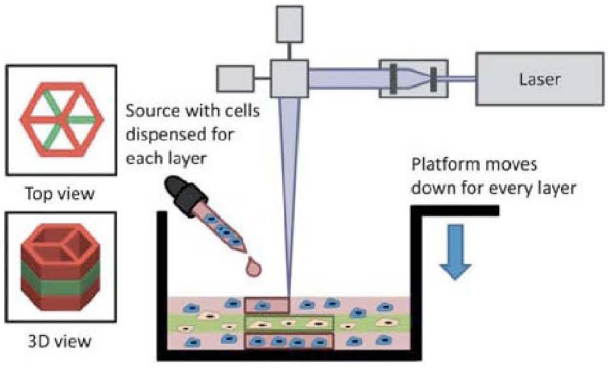

Figure 2. A schematic image of the SLA. The prepolymer solution is pipetted into the container one layer at a time from the bottom to the top. This setup was modified especially for cell encapsulation applications, which required a reduction in total volume of photopolymer in use and removal of photopolymer from static conditions that cause cells to settle [2].

II. 3D Printing with Stereolithography

We begin by identifying a 3D printer that could be adapted for printing living cells and biomaterials. Since 3D printers use a variety of fabrication methods (*i.e.* heat, light, adhesives, etc.), it is important to choose a 3D printer that is biocompatible and can be processed under mild conditions. We demonstrated that a stereolithography apparatus (SLA) can build complex 3D micro environments using hydrogels that support living cells (**Figure 2**).[2] The SLA uses a UV laser (λ = 325 nm) to photopolymerize light-sensitive materials. Computer-controlled scanning mirrors are used to direct the laser onto the surface of the material in a predefined pattern. The blueprint for this pattern comes from a 3D computer-aided design (CAD) model that is sliced into

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2D cross-sections. Once the first layer is photopolymerized, a new layer of light-sensitive material is added, and the process is repeated to completion.

Poly(ethylene glycol) (PEG) diacrylate, a photo-polymerizable hydrogel, was used as the backbone of our biomaterial not only because of its inherent hydrophilicity and biocompatibility, but also its flexibility in functionalization and use. The effect of PEG chain length, for example, can alter the swelling behavior and mechanical properties of the 3D micro environment in a coupled but inverse relationship. As a comparison, PEG Mw 700 g mol^{-1} had a small average pore size (3 nm) and high elastic modulus (500 kPa), while PEG Mw 10,000 g mol^{-1} had a large average pore size (20 nm) and low elastic modulus (5 kPa). Fibroblasts (NIH/3T3 cell line) encapsulated in these hydrogels survived the SLA processing conditions and continued to live on for well over 2 weeks in PEG chain lengths ≥ 3,400 g mol^{-1} (almost 50% survival). The addition of adhesive RGD peptide sequences further increased viable cells, and promoted their spreading and proliferation (over 6-fold increase).

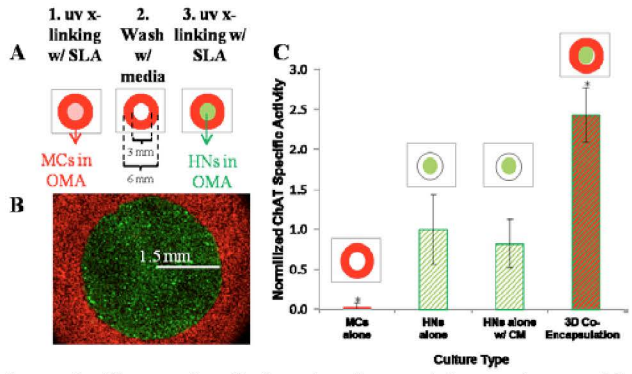

Figure 3. Neuronal cell function in spatial co-culture with muscle cells. (A) Schematic outline of SLA fabrication process with muscle cell toroid and neuronal cell cylinder. (B) Fluorescence image of neuronal cells (green) in the center of a toroid of muscle cells (red). (C) Choline acetyltransferase (ChAT) activity of neuronal cells in different culture conditions after 10 days. CM stands for conditioned medium, $n = 6$. $*$ indicates the significance ($p < 0.001$) [3].

III. Multi-Cellular Constructs

The number of different building blocks that make up our 'toolbox' for creating bio-machines is dependent on the number of specialized cell types available and the instructive 3D micro environments that define their function. In addition to fibroblasts, we tested a variety of other cell types, such as muscle cells, neuronal cells, and even stem cells, for their survival and functionality under SLA processing conditions.[3] We also enhanced the backbone of our PEG biomaterial by incorporating a degradable oxidized methacrylic alginate (OMA) to PEG Mw 1,100 g mol^{-1}. Muscle cells (C2C12 cell line), neuronal cells (rat hippocampal and PC12 cell line), and stem cells (porcine adipose-derived) were encapsulated in these hydrogels and exhibited a similar if not improved percentage of viable cells over 2 weeks (almost 100% neuronal cells survived). When muscle cells were co-cultured with neuronal cells in a single construct, an increase in cholinergic function of the neuronal cells was observed by evaluating choline acetyltransferase (ChAT) activity (over 2.5-fold increase) (**Figure 3**). This increase in ChAT activity was not observed when neuronal cells alone were cultured in conditioned muscle cell medium.

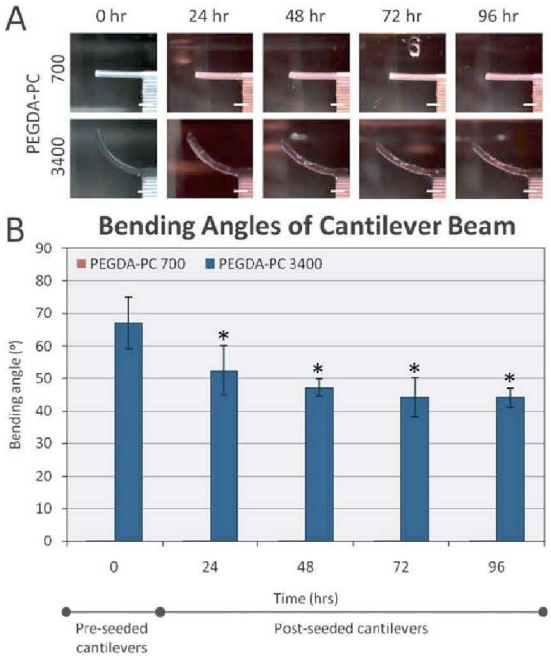

Figure 4. Cell sheet stress calculations. Cells from the ventricles of neonatal rat hearts were seeded on the backside of the cantilever beams. (A) The traction forces of these cells, which are responsible for migration, proliferation, and differentiation, caused the PEG Mw 3400 g mol^{-1} cantilever beams to deflect downward in the Z-direction over time. (B) The average bending angles of the cantilevers were measured over a 96 hour period, which was used to calculate the deflection at the tip of the beams. Scale bars are 1 mm. Statistics by one-way ANOVA, Tukey's test, $*p < 0.05$ for $n = 8$ and SD [4].

IV. Multi-Material Constructs

In addition to encapsulating multiple cell types in a single construct, the SLA is also capable of multi-material fabrication. This is a key feature for tuning physical and chemical cues of the 3D micro environment for a particular cell type, while retaining our ability to combine these building blocks into a single, integrated construct, our 'bio-machine'. The physical cues, such as geometry and stiffness, and chemical cues, such as adhesive groups and growth factors, can be controlled spatially through the SLA. For instance, we printed 3D bioactuators using two different stiffnesses of hydrogels, a rigid base with PEG Mw 700 g mol^{-1} (500 kPa) and a soft cantilever with PEG Mw 3,400 g mol^{-1} (30 kPa).[4] The cantilever was tuned to the elastic properties of cardiac muscle cells from rat to maximize contractile forces per cell sheet (5 μN) and cantilever actuation (400 μm). Again, the PEG backbone of the biomaterial was modified to help cells perform their designated function. In this case, acryl-collagen was

incorporated into the PEG backbone to enhance cardiac muscle cell adhesion, spreading, and organization. Cardiac muscle cells seeded on the cantilevers generated cell traction forces (passive tension) and contractile forces (action tension), which were calculated using finite element simulations and validated with classical beam equations (**Figure 4**).

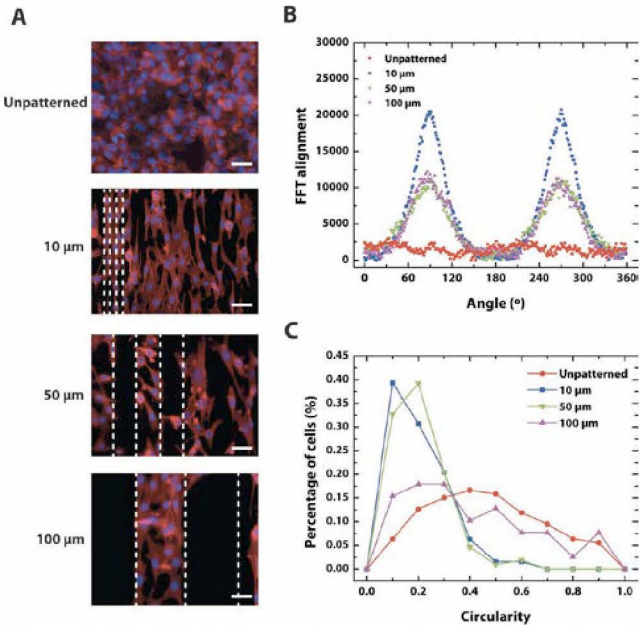

Figure 5. Fibroblast alignment on unpatterned and patterned hydrogels. (A) Fibroblasts with fluorescent nuclear (DAPI) and actin (rhodamine-phalloidin) stains were cultured on hydrogels with unpatterned and patterned acryl-fibronectin with lines 10, 50, and 100 mm wide. (B) Power spectrum generated by radially summating frequencies from fast Fourier (FFT) converted images. Peaks at 90° and 180° correspond to vertical alignment in images and can be seen for fibroblasts patterned on lines. (C) Analysis of fibroblasts at the cellular level shows a significant decrease in circularity for cells grown on 10 and 50 mm lines. A small but insignificant shift can be seen on 100 mm lines. A decrease in circularity can be attributed to a constriction of cellular growth area. Scale bars represent 50 mm wide [5]

V. Instructive 3D Micro Environments

Instructive 3D micro environments that guide cell growth and form patterns are critical to the functional components of bio-machines. Motor neurons can be directed to align and interact with muscle cells to form functional neuromuscular junctions (NMJs). Contractile muscle cells can be aligned to improve differentiation (such as sarcomere formation) and generate larger contractile forces. To do this, we demonstrated a simple method that aligned cells on our 3D printed hydrogels by combining micro-contact printing (µCP) with the SLA.[5] First, fibronectin modified with acrylate groups were printed on glass coverslips with unpatterned, 10, 50, and 100 mm wide line patterns. Then, the patterns were transferred to 3D printed hydrogels through chemical linkages during photopolymerization in the SLA. Fibroblasts (NIH/3T3 cell line) cultured on protein-transferred 3D hydrogels aligned in the direction of the patterns, as confirmed by fast Fourier transform and cell morphometrics (**Figure 5**). Other cell types, such as the motor neurons and muscle cells mentioned earlier, can be cultured using this same method.

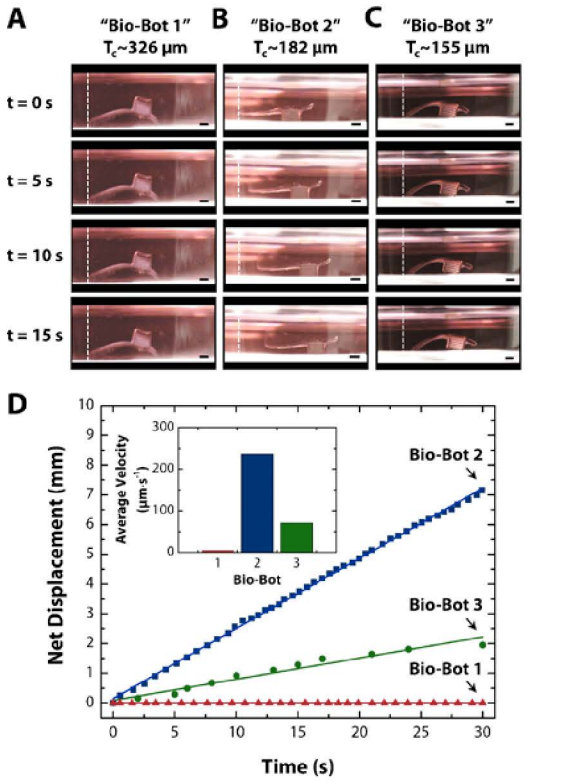

Figure 6. Demonstrations of walking bio-machines. (A-C) Time course of net forward motion for "bio-bot 1" (326 µm thick), "bio-bot 2" (182 µm thick), and "bio-bot 3" (155 µm thick) over 5 second intervals for a period of 15 seconds. (D) Plot of net displacement vs. time for all three bio-bot designs. The inset is a plot of average velocity vs. bio-bot design, which is extracted from the plot of net displacement vs. time (Scale bars: A-C, 1 mm.) [6].

VI. 3D-Printed Walking Bio-Machines

Here, we demonstrate proof-of-concept of a 3D-printed, walking bio-machine based on the autonomous contraction of cardiac muscle cells.[6] The multi-material bio-machine consisted of a 'biological bimorph' cantilever structure as the actuator to power the bio-bot, and a base structure to define the asymmetric shape for locomotion. The cantilever structure was seeded with a sheet of contractile cardiac muscle cells on a variably-thick hydrogel layer. The thickness of the hydrogel layer and cytoskeletal tension from the cell sheet on the collagen-functionalized hydrogel determined the degree of curvature of the cantilever structure. We evaluated the locomotive mechanisms of several designs of bio-bots by changing the cantilever thicknesses. The bio-bot that demonstrated the most efficient mechanism of locomotion maximized the use of contractile forces for overcoming friction of the supporting leg, while preventing backward movement of the actuating leg upon relaxation. The maximum recorded velocity of the bio-bot was ~236 $\mu m \cdot s^{-1}$, with an average displacement per power

stroke of ~354 µm and average beating frequency of ~1.5 Hz (**Figure 6**).

VII. Conclusion and Future Directions

Many challenges exist towards the development of cell-based biological systems and machines. Formation of multi-cellular clusters, both homotypic and especially heterotypic, requires careful optimization of growth and culture conditions so that functional interactions can be designed and used to create specific functionalities. Similarly, the creation of these clusters in 3-D environmentally instructive matrices with controlled mechanical and chemical stimuli is another important requirement for such cellular systems.

Acknowledgment

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