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Biomimicry, Biofabrication, and Biohybrid Systems: The Emergence and Evolution of Biological Design

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The discipline of biological design has a relatively short history, but has undergone very rapid expansion and development over that time. This Progress Report outlines the evolution of this field from biomimicry to biofabrication to biohybrid systems' design, showcasing how each subfield incorporates bioinspired dynamic adaptation into engineered systems. Ethical implications of biological design are discussed, with an emphasis on establishing responsible practices for engineering non-natural or hypernatural functional behaviors in biohybrid systems. This report concludes with recommendations for implementing biological design into educational curricula, ensuring effective and responsible practices for the next generation of engineers and scientists.

1. Introduction

Dynamic problems require adaptive solutions. Since most engineered systems function in environments with constantly variable conditions, there is a great need for responsive systems that can adjust and perform in new surroundings. This is the underlying motivation for utilizing the responsive biological materials that make up the natural world. Dynamically adaptive functional behaviors such as self-assembly, self-healing, and environmental adaptation are inherent to biological materials. The discipline of biological design encompasses understanding of the mechanisms of these adaptive behaviors, and utilizing these capabilities in forward design of synthetic, natural, and biohybrid systems.

Biological design has a relatively short history, but has undergone very rapid expansion and development over that time. In this progress report, we will outline the evolution of this field from biomimicry to biofabrication to biohybrid systems, showcasing how the need for engineered environmental adaptation is prevalent in all subfields. First, we shall trace how the concept of bioinspiration, or imitating biological design and functionality in synthetic materials, created a new class of "smart" biomimetic materials. Then, we shall discuss how the continuing development of enabling manufacturing technologies, such as 3D printing and microfluidics, has established the separate subdiscipline of biofabrication for tissue engineering, or "building with biology." Finally, we shall investigate the convergence of these two fields into the emerging discipline of biohybrid design, the use of biological materials to power non-natural functional behaviors in synthetic machines.

Throughout this report, we will revisit a single class of materials, hydrogels, as a case study of biological design in the context of each subfield, and discuss how the constraints, underlying principles, and end-use applications differ in each case. We will also discuss ethical considerations of biological design, with a special focus on forward engineering non-natural or hypernatural functional behaviors in biohybrid systems. We will conclude with recommendations for implementing biological design into educational curricula, ensuring effective and responsible practices for the next generation of engineers and scientists.

2. Biomimicry and Bioinspired Design

A deeper understanding of the underlying design principles that govern biological systems has inspired the field of biomimicry.[1,2] Observing adaptive phenomena in nature, scientists and engineers have sought to extract the components of biological design responsible for this behavior and replicate the behavior in synthetic materials. Fundamentally, this involves understanding the building blocks or base units from which a biological material is built, the hierarchical assembly of these building blocks, and the interactions and interfaces between them.

In this section, we will discuss strategies that engineers and scientists have employed to engineer bioinspired hierarchy, from bottom-up self-assembly and top-down engineered assembly. We will present several key demonstrations of stimulus-responsive hydrogels ranging from the micro- to the macroscale, and investigate novel demonstrations in biomimetic actuation and movement. We will conclude with the remaining challenges in the field of biomimicry and discuss the potential future impact of bioinspired design.

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2.1. Engineering Bioinspired Hierarchy

The chief prerequisite to designing a biomimetic material is a fundamental understanding of the function of the biological systems by which it is inspired. As this is the aim and responsibility of the discipline of biology, it is not within the scope of this report on biological forward design. Assuming, however, that this fundamental understanding is present, the next step is to extract the functional parts of the natural systems, and assess a manufacturing approach and base synthetic material with which to replicate adaptive functionality.

During this stage of design, scale is a key factor. The base functional units of biological systems, living cells, are generally on the order of 1–100 µm, and the functional units within cells, proteins, are nanoscale. It is thus unsurprising that the rise and widespread popularity of micro- and nanoscale fabrication across many field of science and engineering has inspired mimicking biological design at this scale.[3] By removing, or at least mitigating, the difficulty of replicating small-scale features at the sizes in which they are present in natural systems, microfabrication and nanofabrication have been fundamental toward the creation of smart synthetic materials.

While manufacturing imitations of functional base units, or building blocks, can be readily accomplished in most cases, there exists an unresolved dichotomy in the approach used to assemble the units into a functional whole. Biological systems rely on an autonomous process, namely self-assembly, to hierarchically organize these building blocks and coordinate communication between them. Mimicking biological materials is, however, an inherently top-down approach that relies on reverse engineering adaptive functionality. This type of engineered assembly treats each subunit as a "black box," where the form and composition of the box are less important than the function it performs. In this section, we will investigate the motivations underlying both self-assembly and engineered assembly, and present significant recent advancements generated by both approaches.

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2.1.1. Self-Assembly of Bioinspired Materials

By definition, self-assembly requires that the design of the building blocks that make up a system autonomously drives formation from a disordered grouping into a functional whole and that this formation be reversible.[4] Again, novel fabrication approaches that allow for morphological control and patterning of surface properties are crucial to designing such building blocks and forward engineering their interactions with each other.

One promising approach toward self-assembly is self-folding, essentially engineering the phenomena of autonomous origami. In general, these approaches rely on incorporating internal stresses within flexible materials and using these stresses to transform 2D material sheets into 3D structures.[5] Hydrogels, the soft hydrophilic polymers that we will use as a case study throughout this report, have tunable mechanical properties that can be modulated by chemical composition and crosslinking density.[6] This renders them an ideal base material for tuning internal stresses, as spatially segregating the degree of swelling in different regions of the hydrogel generates such stresses in a precise and controllable manner.[7]

Gracias and co-workers have utilized this principle of patterned differential swelling to drive self-folding of poly (ethylene glycol) (PEG)-based hydrogel bilayers.[8] Their approach, relying on conventional photolithography to pattern PEG-based hydrogels, has broad applicability toward the manufacturing of microscale and macroscale 3D geometries. Precise tuning of hydrogel molecular weight and thickness in each layer allowed for the patterned self-folding of a range of geometries, including spheres, helices, and cylinders. Gracias and co-workers also demonstrated that each of these structures could be designed to include microscale surface features, such as posts and holes, which could be used to regulate interactions and interfaces with other base units of an assembly.

An important criterion of self-assembly is, of course, that the autonomous formation of a functional whole should be reversible. Yang and co-workers have demonstrated pH-triggered reversible self-folding of hydrogel bilayers into hollow

microscopic spheres, with potential applications as drug delivery devices or microrobots.[9] These spheres are composed of an active hydrogel layer, poly(2-hydroxyethyl methacrylate-*co*-acrylic acid), and a passive hydrogel layer, poly(2-hydroxyethyl methacrylate). At basic pH levels, the active layer swelled, forming a closed hollow microsphere. Reducing pH triggered a decrease in the degree of swelling demonstrated by the active layer, opening the microspheres and releasing internal contents.

The most successful approaches toward self-assembly of synthetic materials and systems rely on imitating assembly processes and geometries present in nature, such as spheres and helices. These have served to demonstrate that autonomous, reversible, and precisely controllable formation of a functional whole from a set of base units can be forward engineered, matching the criteria imposed by the definition of self-assembly.

2.1.2. Engineered Assembly of Bioinspired Materials

The engineered assembly of adaptive materials draws inspiration on base unit structure from biological systems, but relies on both bioinspired and man-made manufacturing approaches to combine the base units into a functional whole. A promising approach toward engineered assembly is based on shape memory polymers, which depend on external cues, such as heat- or electrical activation, to trigger conformational changes between stable states.[10] These shape memory polymers can be assembled into complex 3D structures using manufacturing approaches such as 3D printing and textile-inspired fiber weaving and knitting.[11–13]

Returning to the case study of hydrogels, there have been several significant examples of shape memory hydrogels in recently published literature. Osada and Matsuda demonstrated that heating and cooling of a thermo-responsive polymer, formed by co-polymerization of acrylic acid and *n*-stearyl acrylate, could be used to mimic shape memory behaviors in hydrogels.[14] When heated, the polymer became soft and flexible, allowing for ready deformation into complex 3D shapes. Subsequent cooling of the polymer increased rigidity, promoting retention of the 3D shape despite the removal of external forces driving deformation. Hao and Weiss have built on this work by developing a thermoresponsive shape memory hydrogel that switches between two flexible states, allowing for exploitation of soft and hydrophilic material properties both below and above the switching temperature.[15] This quad-polymer material was shown to effectively fix and recover shape, triggered by changes in temperature, and retain flexibility, hydrophilicity, and mechanical toughness throughout. Li and co-workers have extended the range of potential transition mechanisms in shape memory hydrogels by incorporating light and pH-triggered switches, in addition to thermally triggered switches, into their materials.[16] This allows for dual and triple shape memory effect, enabling reversible switching between several stable states.

Shape memory hydrogels serve as significant demonstrations of engineered assembly in smart synthetic materials, and they can be combined with other approaches to further increase the complexity of functional output behaviors. For example, supramolecular interactions have been used to expand the biomimetic capabilities of such materials by giving them the ability to rapidly self-heal and exhibit high mechanical strength in addition to demonstrating shape memory.[17,18] The triggered changes in conformation and functional behavior demonstrated in engineered assembly approaches are also reversible, similar to the self-assembly approaches described above, providing an attractive alternative for manufacturing more complex 3D structures.

2.1.3. Interfaces in Bioinspired Materials

Both self-assembly and engineered-assembly approaches depend heavily on understanding and designing interfaces between building blocks, ensuring coordinated interaction and functional output. Often, this is accomplished by combining building blocks with different surface functionalization and properties.[19] This provides each base unit with a specific place and function within the whole, and also allows for the feedback between units that are essential for reversible assembly and disassembly, and adaptation to dynamic environmental cues.

2.2. Bioinspired Environmental Feedback and Adaptation

The primary motivation for designing bioinspired materials is to replicate their ability to adapt to constantly changing environments. This involves understanding and utilizing the internal and external feedback loops inherent within biological systems. Some of the greatest strides in engineering adaptation into synthetic materials have been demonstrated in hydrogels, perhaps because the porous structure and diverse chemical compositions of these materials make them especially responsive to a range of environmental conditions.[6] Programing specific mechanical and biochemical behaviors within hydrogels is simply a question of regulating the design of the polymer backbone and the mode of synthesis.[20] In this section, we will discuss bioinspired approaches to engineering environmental adaptation in synthetic materials, with a focus on forward design of biomimetic behaviors in smart hydrogels.

2.2.1. Stimulus-Responsive Hydrogels within Microfluidic Systems

Microfluidic devices allow for precisely controllable flow and delivery of liquids to embedded materials and systems, rendering them uniquely suited to studying the response of hydrogels to environmental stimuli.[21] A variety of approaches employing chemical, mechanical, optical, electrical, and thermal stimuli to trigger responsive functional behaviors in smart hydrogels have been demonstrated within microfluidic devices.

Early studies in this field employed responsive hydrogels as valves for controlling flow inside microfluidic systems. Jo and co-workers photopolymerized microscale hydrogel cylinders within microfluidic channels that are capable of reversible expansion and contraction in response to environmental pH.[22] This tunable swelling allowed them to open and close

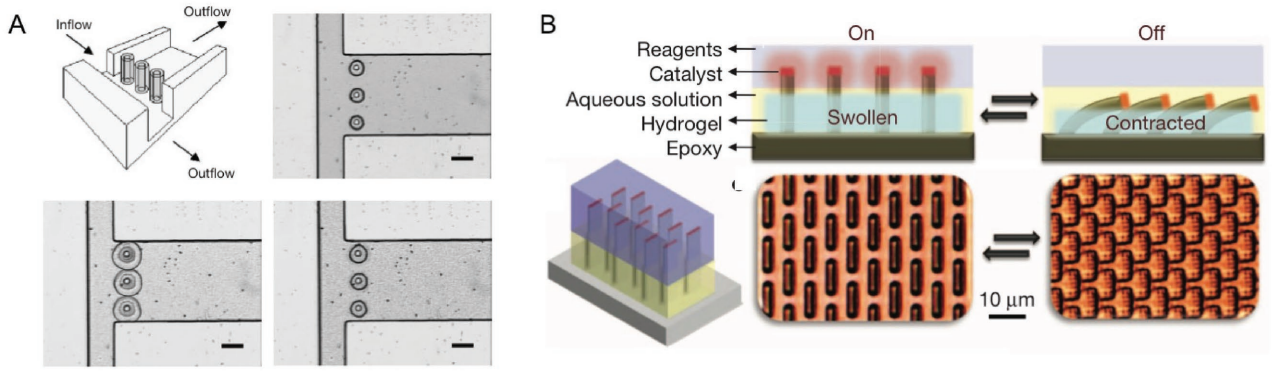

Figure 1. A) Hydrogel-coated microposts in a microfluidic device can reversibly swell and contract in response to pH stimuli, providing a method of controlling flow through microfluidic channels. Scale bars, 300 μm. Reproduced with permission.[22] Copyright 2000, Nature Publishing Group. B) Microscale pillars manufactured from a chemical stimulus-responsive hydrogel reversibly switch between upright and bent states on command. Reproduced with permission.[27] Copyright 2012, Nature Publishing Group.

channels autonomously, enabling the design of a self-regulated flow sorter (**Figure 1**A). Similar microfluidic valves have also been demonstrated by West and co-workers, who demonstrated optical control over swelling in gold-colloid composite hydrogels.[23] This allowed for greater spatiotemporal precision and more independent external control of valve opening and closing. Smart hydrogel components for microfluidic flow control have also been engineered to respond to other forms of external control, such as electrical and thermal stimuli.[24,25]

Stimulus-responsive swelling can also be used to accomplish different functional tasks, such as the formation and adaptive focusing of a liquid microlens. Jiang and co-workers have engineered a microfluidic system in which a stimulus-responsive hydrogel is used as a container for a liquid droplet.[26] This droplet functions as a liquid lens, and the shape of the droplet alters the focal length of the lens. Tuning the swelling behavior of the hydrogel, therefore, regulates the shape of the droplet, providing a precisely triggered and controllable mechanism for adjusting focal length. Jiang and co-workers have demonstrated autonomous focusing triggered by temperature and pH stimuli. This system, when integrated with other microscale components within a lab-on-a-chip device, could prove useful for sensing and medical diagnostics.

Specific applications of smart hydrogels within microfluidic devices for high-throughput biological assays have already been demonstrated. Inspired by the homeostatic abilities of biological systems, Aizenberg and co-workers have developed a hydrogel system with an internally modulated chemomechanical feedback loop that allows for self-monitoring and regulation.[27] This system, termed self-regulated mechanochemical adaptively reconfigurable tunable system, is composed of an array of microscale hydrogel pillars, or microfins, immersed in a liquid bilayer (Figure 1B). Chemical stimulation of a catalyst in the top layer results in a change in the swelling properties of the hydrogel, "turning on" or triggering a conformation change in the microfins from an upright to a bent state. This mechanical deformation brings the catalyst to the bottom layer, turning off the chemical reaction and returning the microfins to their straightened state. This system can be adapted to respond to a range of different environmental triggers, such as pH and temperature. It is also readily controllable within a microfluidic device, enabling a variety of applications. For example, Aizenberg and co-workers used a microfin array of a pH-responsive hydrogel, poly(acrylamide-*co*-acrylic acid), within a microfluidic device to capture biomolecules of interest from a liquid sample.[28] In the upright state, the microfins, which are functionalized with a DNA aptamer, capture target biomolecules flowing through the top layer of the liquid bilayer. When triggered with an acidic solution in the bottom layer, the microfins bend, denaturing the aptamer and releasing the captured biomolecules. This conformational change is reversible, as the microfins can revert to their upright state when immersed in a neutral pH solution. This demonstration of controllable and reversible actuation, with a specific target application, is a powerful example of engineering dynamically responsive functionality in a synthetic material system.

2.2.2. Stimulus-Responsive Hydrogels within Macroscale Systems

Adaptive hydrogel materials have also been applied within macroscale systems, with key progress made within biological systems and in vivo models. Gracias and co-workers have, for example, used smart hydrogels to engineer soft microgrippers for surgical tissue excision and biopsy applications.[29] In this system, the thermoresponsive hydrogel poly (*N*-isopropylacrylamide-*co*-acrylic acid) is embedded with iron oxide nanoparticles to render it magnetically responsive. The hydrogel is then engineered into a microgripper that can be guided to a site of interest with magnetic stimuli, and reversible actuated from its open to its closed state using thermal stimuli (**Figure 2**A). Use of these microgrippers to excise live cells from a tissue clump has been demonstrated, setting the stage for untethered control over microscale surgical robotics in the future.

Stimulus-responsive hydrogels have already been utilized successfully in vivo for applications in drug delivery. Langer and co-workers have developed a supramolecular polymer gel, combining poly(acryloyl-6-aminocaproic acid) and poly(methacrylic acid-*co*-ethyl acrylate), capable of tunable elasticity in response to an external pH stimulus.[30] These gels retain their morphology and elasticity at acidic pH levels, as found in the stomach, and dissolve in neutral pH environments, as found

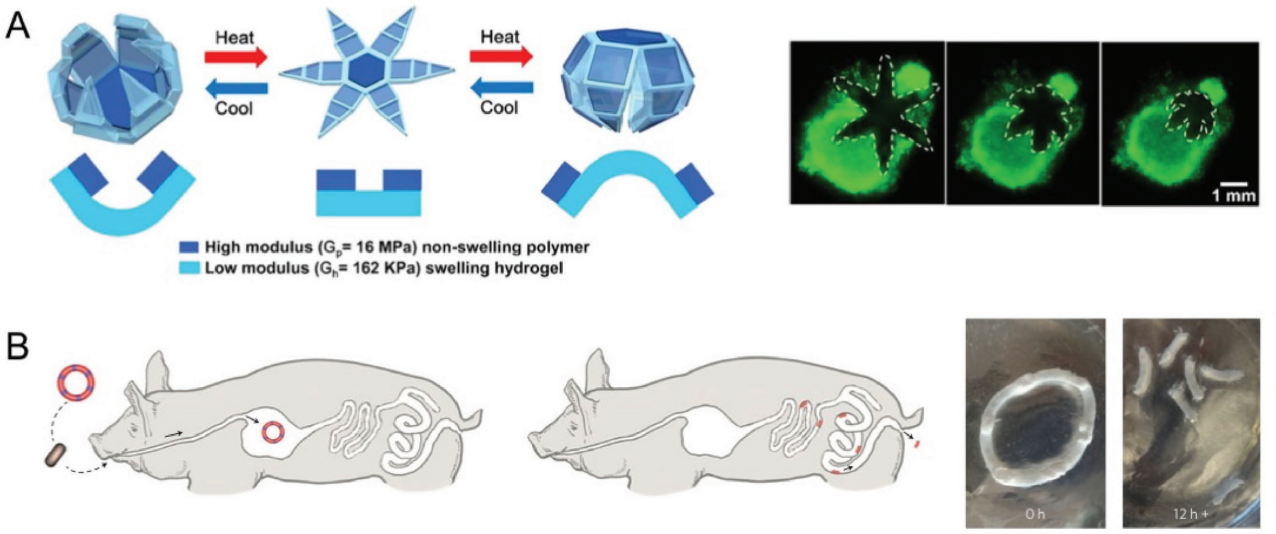

Figure 2. A) Temperature-controlled swelling of a hydrogel is used to drive actuation of a microgripper (left) that can be used to excise live cells (right). Reproduced with permission.[29] Copyright 2015, ACS. B) A pH-responsive supramolecular polymer gel is used to fabricate drug delivery devices that accomplish long-term residence in the acidic environment of the stomach and disintegrate in the neutral pH environment of the intestine, ensuring safe passage. Reproduced with permission.[134] Copyright 2015, Nature Publishing Group.

in the intestine. These hydrogels are thus extremely useful for applications in oral drug administration, as they can reside in the stomach and deliver drugs for extended periods of time, and then dissolve in the intestine, allowing for safe passage (Figure 2B). Langer and co-workers have used this capability of pH-triggered dissolution to engineer a drug delivery platform targeted toward the elimination of infectious diseases, such as malaria.[31] They have engineered a device that is swallowed as a capsule and unfolds into a star-shaped structure in the stomach, allowing for prolonged gastric residence by preventing passage through the pylorus.[134] After delivering drugs over a period of several weeks, the pH-responsive components of the star-shaped structure dissolve, allowing safe passage through the intestines.

2.2.3. Stimulus-Responsive Hydrogels for Biomimetic Actuation and Movement

Actuation is perhaps the most popular biomimetic behavior that engineers have attempted to replicate in smart synthetic systems. Movement in biological systems is not dependent upon the rigid joints and fixed degrees of freedom typical to movement in man-made machines, but is rather centered on flexibility and compliance. This allows for tunable response and adaptation to unpredictable and changing environments, and allows for much more complex functional tasks such as triggered actuation and autonomous navigation.[32]

Adaptive response in a synthetic material requires controllable and predictable conformational changes in response to external stimuli. The flexibility and tunable chemical compositions of hydrogels, as demonstrated in the previous sections, render them an ideal class of materials for applications in biomimetic actuation as well. Presented below are a few key examples of biomimetic movement in soft robots enabled by the compliant and stimulus-responsive properties of smart synthetic hydrogels.

Hashimoto and co-workers engineered autonomous locomotion in a poly(NIPAAm-*co*-Ru(bpy)3-co-AMPS hydrogel robot, relying on the cyclical swelling–deswelling properties of the gel to drive worm-like crawling of the robot across a surface.[33] Stimulus-responsive motility has also been engineered in such robots by Hori and co-workers, who imitated worm-like locomotion in an electrically triggered poly(2-acrylamido-2-methyl propane) hydrogel.[34]

In addition to bioinspired approaches for stimulus-responsive or autonomous actuation of smart synthetic hydrogels, there has also been significant interest in drawing design inspiration for robots from natural biological systems. For example, Zhao and co-workers have recently imitated the biological design of a sea creature, the leptocephalus, in hydrogel robots composed of polyacrylamide-alginate hydrogels.[35] These robots rely on hydraulic actuation to produce a range of complex functional behaviors, including swimming, kicking, and gripping (**Figure 3**A). Their chemical composition also allows for optical transparency and sonic camouflage in water, demonstrating the range of multifunctional behaviors that can be produced by biomimetic actuating machines.

Recent advances have also combined biomimetic materials with existing design components typical to man-made systems, such as gears, valves, and rotors. Sia and co-workers have, for example, engineered hydrogel-based microdevices with gears and manifolds that can be controlled via external magnetic stimuli and used as implantable drug delivery reservoirs (Figure 3B).[36] Macroscale structures mimicking synthetic machines have also been designed, such as the functional loudspeakers engineered by Suo and co-workers.[37] Relying on the ionic conductive and elastomeric properties of a polyacrylamide based hydrogel, they showed that voltage stimuli could be used to generate sound spanning the audible range of frequencies.

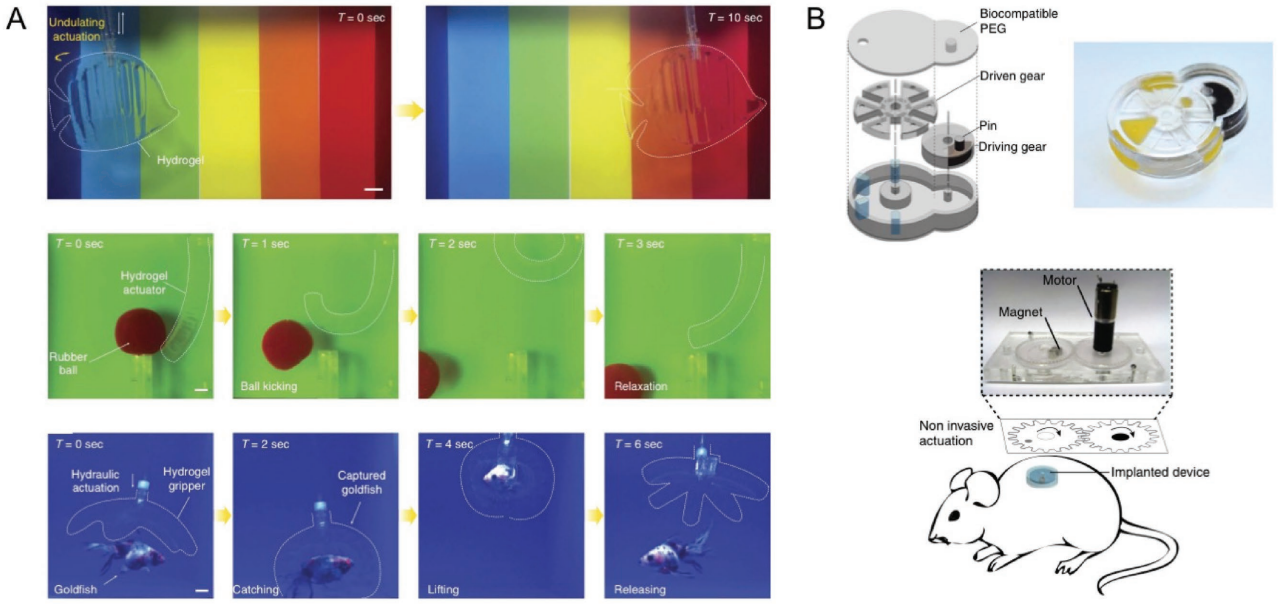

Figure 3. A) Hydraulic actuation of optically transparent hydrogel actuators is used to generate functional behaviors such as swimming (top), kicking (middle), and gripping (bottom). Reproduced with permission.[35] Copyright 2017, Nature Publishing Group. B) Millimeter-scale hydrogel devices that incorporate functional elements, such as magnetically actuated gears, can serve as implantable drug delivery reservoirs in vivo. Reproduced with permission.[36] Copyright 2017, AAAS.

These demonstrations, among others, showcase the future potential of smart synthetic hydrogel-based machines and prove their ability to accomplish actuation and movement in response to precisely controllable external stimuli.

2.3. Remaining Challenges and Future Impact

In this section, we have outlined several promising approaches toward engineering bottom-up hierarchy in bioinspired systems and enabling top-down control over their functional behaviors, and adaptive response to dynamic environmental cues. A remaining challenge applicable to all subfields of bioinspired materials design is the technical difficulty of fabricating complex 3D multimaterial structures.[3,38] Building systems composed of functional materials with a broad range of chemical and mechanical properties, with features ranging from the nano- to the macroscale, require robust, rapid, and cost-effective manufacturing tools and practices. A coordinated multidisciplinary effort will, therefore, be required to advance the state-of-the-art in this field. Despite this, significant advances in this field have served as proof of the potential impact of biomimicry and bioinspired design.

3. Biofabrication

Advances in manufacturing have enabled significant progress in mimicking biological structures and function in synthetic materials, but they have also driven developments in replicating biological structures and function using biological materials. The idea of "reverse engineering" nature using natural materials is the core philosophy underlying the field of biofabrication.

In this section, we will discuss about enabling technologies for biofabrication, including microfluidics and 3D printing, as well as a range of applications in tissue engineering and regenerative medicine. We will also discuss how reverse engineered models of biological systems have served as fundamental platforms for understanding the signal cascade of environmental feedback and adaptation in such systems.

3.1. Enabling Technologies

Manufacturing multiscale structures using biocompatible and biological materials is a huge technical challenge, and this requires a range of enabling technologies to render it possible. Microfluidic systems and 3D biofabrication apparatus have perhaps been the most widely used and well-developed tools for biofabrication, as they are reliable, replicable, and widely applicable to a range of biomaterials.

Once again, we will focus on demonstrations of fabricating hydrogels with these systems. Hydrogels have special relevance in biofabrication since their mechanical and hydrophilic properties mimic those of biological tissue and organs. Moreover, as seen in the previous section, the readily tunable chemical structure of hydrogel networks makes it relatively easy to integrate biological moieties into the polymer backbone. Advances in chemical synthesis approaches, such as click chemistry and supramolecular hydrogels, have provided a strong platform for synthesis, patterning, and controlled degradation of hydrogels in response to environmental stimuli.[39,40] Hydrogel systems are, therefore, ideally suited to serve as support scaffolds or extracellular matrix mimics for living cells cultured in 3D in vitro environments.

3.1.1. Microfluidic Systems for Biofabrication

Microfluidic systems present an attractive environment for synthesis of biofabricated systems, as they enable precise control over environmental cues and high-throughput monitoring of signals and responses with high sensitivity. Moreover, fabrication processes for microfluidics are well characterized and span an increasingly broad range of structures and functions including on-board pumps, valves, and mixers.[21] These mechanisms, when coupled with the advantages of hydrogels as scaffolds for living cells, generate controlled microenvironments for cells that closely replicate their in vivo environments.

Kamm and co-workers have demonstrated a microfluidic platform for studying cellular dynamics within 3D hydrogels, with real-time monitoring of the adaptation of cellular functions in response to changing environmental cues.[41] The central portion of their microfluidic platform consists of a "cage" patterned with an array of microposts. This is intended to serve as a housing and support for hydrogels and cells that are microinjected into the gel cage. Flow in the cage is controlled via two parallel channels, which mimic forces imposed by interstitial and surface shear flow, and cellular activity is monitored via a microscope. They have demonstrated the efficacy of this approach in monitoring lumen formation in microvascular endothelial cells, but the central platform serves as a model for culturing and observing a range of cell types in controlled 3D environments.

Hydrogels can also be formed in more controlled architectures by replacing a direct injection approach with a multilayered fabrication methodology. For example, West and co-workers have demonstrated a multilayered molding technique that enables embedding PEG diacrylate hydrogels within microfluidic housings manufactured independently using poly(dimethyl siloxane) (PDMS).[42] This approach allows for more precise control over hydrogel architecture, and perfused flow through these scaffolds has been shown to increase viability and prolong metabolic activity of embedded cells. Alternatively, cells and hydrogels can be patterned within microfluidic devices using a combination of optical tweezers and photolithography. This was demonstrated by Timp and co-workers, who manipulated individual cells to specific regions of a microfluidic device using optical tweezers, and then fixed them in place by flowing and polymerizing a light-sensitive hydrogel around them.[43] This approach allows for extremely precise patterning and assembly of cells into microscale tissues on a chip, but can prove to be time-consuming for tissues composed of hundreds or thousands of cells, as each cell requires individual placement. More high-throughput patterning and alignment can be accomplished via the integration of microscale topographical cues within microfluidic devices. Yu and co-workers have developed a method for the incorporation of microgrooves into microfluidic channels, and shown that the grooves serve as cues for the alignment and enhance differentiation of skeletal muscle cells.[44]

Microfluidic devices have also been used to manufacture cell-laden hydrogels that are then cultured outside microfluidic environments. Khademhosseini and co-workers used a stop-flow lithography approach within a microfluidic channel to make microscale hydrogel blocks contain embedded cells.[45] These types of cellular "building blocks" can be assembled or molded following manufacture into complex 3D shapes, as demonstrated by Takeuchi and co-workers.[46] Lee and co-workers have designed a microfluidic-based biofabrication tool that can generate cell-laden microfibers with spatially coded placement of multiple cell types.[47] These types of biological microfibers can be assembled into higher-order structures using microfluidic weaving apparatus, as demonstrated by Takeuchi and co-workers.[48]

Several promising manufacturing approaches for seeding cell-laden hydrogels with and within microfluidic systems have been developed. Depending on the application at hand, a combination of these approaches can be used to study interactions between multiple cell or tissue types, and reconstitute the complexity of in vivo architecture and microenvironment.

3.1.2. 3D Printing Apparatus for Biofabrication

3D printing centers on the idea of building complex 3D structures by sequentially layering 2D cross sections on top of one another. 3D printers take multiple forms, and each form relies on different physiochemical mechanisms to drive formation of solid structures. Two types of 3D printing, stereolithography (SL) and extrusion bioprinting, are most commonly and widely used in the application of biofabrication.

SL is remarkable for its high-resolution fabrication capabilities and ready adaptability to a variety of material types.[49] Stereolithographic processes rely on light to polymerize photosensitive resins from liquid states to gelled solid states.[50] This process is particularly amenable to fabrication with hydrogels, as light-initiated polymerization of such materials is well characterized and understood.[51]

Boland and co-workers demonstrated that the commercial laser-based SL apparatus could be adapted to fabricate scaffolds composed of poly(ethylene oxide) and poly(ethylene glycol) dimethacrylate hydrogels.[52] These hydrogels were shown to mimic the mechanical properties of soft tissues in biological systems, and viable encapsulation of cells within these hydrogels was also demonstrated. Wicker and co-workers subsequently used an SL system to fabricate complex 3D cell-laden structures.[53] We have shown that tuning the biochemical composition of the photosensitive hydrogel resins used by such SL systems can preserve cellular viability and promote metabolic activity over long-term in vitro cell culture.[54]

In addition to laser-based SL systems, significant progress has been made in projection-based apparatus that relies on physical or digital masks to polymerize entire 2D cross sections of a 3D structure within a single exposure.[55] Since laser-based approaches require rasterizing a laser across a surface to create a 2D pattern line by line, projection SL systems can greatly increase throughput by decreasing the fabrication time per part. Chen and co-workers have demonstrated that projection SL apparatus can be used to fabricate hydrogel scaffolds, and that cells seeded on or embedded within these scaffolds can serve as in vitro models of complex biological systems.[56,57] We have shown that such systems can even be used to pattern cells seeded within hydrogels at resolutions <5 μm, on the order of cells and cell signals in vivo, and that printed microscale tissues

remain viable and metabolically active up to 2 weeks postfabrication.[58]

Both laser-based and projection-based SL systems can be integrated with cellular patterning approaches to provide greater control over the placement and interaction of cells seeded within a hydrogel matrix. We have, for example, integrated microcontact printing techniques with SL to pattern and align cells on printed hydrogel structures.[59] We have also integrated dielectrophoresis approaches with SL to pattern cells embedded within printed hydrogel structures.[60] Stereolithography thus provides a versatile baseline platform for fabricating complex 3D architectures from cell-laden hydrogels.

Extrusion bioprinting is another most commonly used form of 3D printing applied to biofabrication. In this approach, liquid mixtures of cells in un-crosslinked hydrogels are deposited layer by layer, prior to curing into a gelled state via various physiochemical processes.[61] Forgacs and co-workers drove early advances in this field by depositing bioink spheroids, composed of multiple cells, onto a biocompatible support structure, termed biopaper.[62] Following printing and postprocessing, these spheroids fused with each other, driving forward assembly of complex 3D structures with high densities of embedded cells. They later adapted this approach by first printing cylinders, composed of several fused multicellular spheroids, and then assembling the cylinders to manufacture complex tubular structures, mimicking a vascular tree.[63] Since patterning vasculature at multiple scales with several different cell types is an ongoing technical challenge in tissue engineering, this technique presented a broadly applicable approach for extrusion bioprinting of vascularized organs.[64]

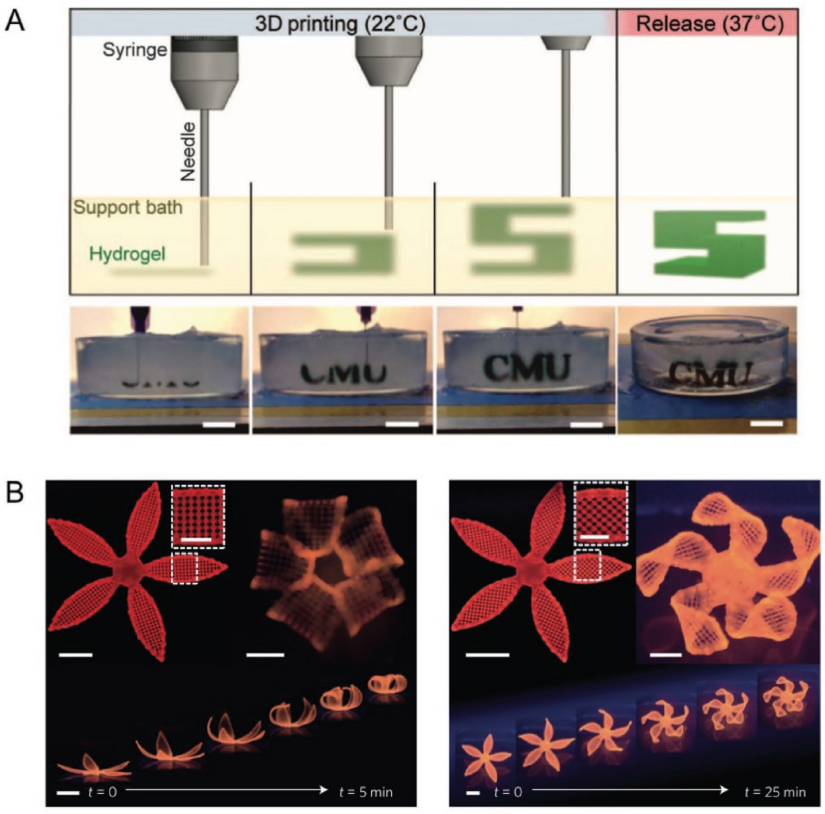

Figure 4. A) Schematic (top) and representative images (bottom) of the FRESH printing process, which relies on extruding hydrogel inks within a support bath, crosslinking the deposited structure, and then melting the support structure. Scale bar, 1 mm. Reproduced with permission.[65] Copyright 2015, AAAS. B) By regulating the orientation of cellulose fibers within an acrylamide matrix, anisotropic swelling of printed structures drives the formation of complex flower-like morphologies. Scale bar, 5 mm; Inset scale bar, 2.5 mm. Reproduced with permission.[66] Copyright 2016, Nature Publishing Group.

The compliant nature of extrusion bioprinting inks, which are composed of cells mixed with un-crosslinked hydrogels, renders it difficult for bioprinters to manufacture large and complex 3D structures mimicking anatomical architecture. Feinberg and co-workers have recently demonstrated an approach that allows for printing large structures from soft bioinks, relying on freeform reversible embedding of suspended hydrogels (FRESH).[65] FRESH printing is accomplished via patterned deposition of hydrogel bioinks within a gelatin slurry bath (**Figure 4**A). The deposited structure is crosslinked within the support structure prior to melting of the support structure at 37 °C. Feinberg and co-workers have used this approach to print a variety of complex anatomical structures, including those found in brains, hearts, and internal vasculature.

Since many biological structures adapt to their surroundings and evolve their geometry with time, Lewis and co-workers have developed a "4D" extrusion bioprinting approach that utilizes time-reliant processes to manufacture complex 3D structures.[66] Their approach that utilizes a composite hydrogel bioink of cellulose fibers in a matrix of acrylamide relies on localized control over the orientation of these fibers within the matrix, which, in turn, leads to anisotropic swelling of the printed bioink when the structure is immersed in water. They have used this technique to manufacture complex geometries inspired by the plant world (Figure 4B). Adaptation of this technique toward mimicking architectures present in human bodies could have significant impact on the field of biofabrication, specifically with regard to clinical applications.

Both stereolithography and extrusion bioprinting have contributed significantly toward advances in biofabrication, as shall be evidenced in the following sections on tissue engineering. Their ability to replicate the form of macroscale anatomical structures, using the biomaterials that give those structures their function in vivo, is critical for applications in regenerative medicine and biofabricated therapeutics.

3.1.3. Novel Manufacturing Approaches

In addition to the adaptation of manufacturing methodologies such as microfluidics and 3D printing to biofabrication,

other approaches have more recently been used for bottoms-up assembly of biological systems. The origami-inspired approaches for self-assembly, presented in Section 2 of this report, have recently been adapted toward biofabrication. Hwang and co-workers have, for example, engineered a foldable paper, laden with biofunctionalized hydrogels, that can be seeded with cells and assembled into 3D structures using origami-inspired methods.[67] Gracias and co-workers have demonstrated that cells can also be embedded within self-folding PEG-based hydrogels prior to assembly into 3D structures, and that multicellular multilayered structures can be generated using this methodology.[8]

Manufacturing techniques inspired by textile weaving have also been explored for their ability to generate complex 3D architectures. Guilak and co-workers drove early advances in this field by developing a weaving technique for generating cartilage tissue from a composite mixture of chondrocytes and hydrogels. This microscale weaving approach enabled them to engineer tissue with anisotropic mechanical properties mimicking those observed in cartilage in vivo.[68] The approach developed by Takeuchi and co-workers, described in Section 3.1.1. of this report, further increased the complexity of tissue weaving by incorporating multiple cell types into 3D constructs. By testing tissue fibers in vivo, they have also been able to demonstrate the safety and feasibility of this approach for medical applications in the future.[48] Recently, Tate and co-workers have proposed an algorithm for weaving biological tissues by mapping the composition and 3D distribution of collagen and elastin fibers in bone and inputting the information into a digital loom.[69] This approach could, by mimicking the 3D micro- to macroscale architecture seen in nature, help replicate the strength, resilience, and lightness of biological systems. It is likely that origami- and weaving-inspired techniques, as well as other manufacturing approaches, will be applied toward biofabrication in the coming years. Each methodology will come with strengths and limitations, ranging from resolution to mechanical properties to cost efficiency and manufacturing time, and it is likely that a multipronged approach will be required to address challenges specific to individual biofabrication applications.

3.2. Medical Applications of Biofabrication

Building biological systems from the bottom-up hierarchy, and ensuring that they can mimic the form and function of systems found in nature, have obvious and important implications for novel therapeutics. Microscale biological systems can be used to study tissue pathology and to conduct high-throughput testing and optimization of drugs. Macroscale systems can be used to replace diseased or damaged tissue in the body, effectively regenerating functional subunits of a complex multipart biological system. We will present microscale organ-on-a-chip systems, embedded within microfluidic devices, and macroscale engineered tissues, fabricated via processes such as 3D printing, and discuss the state-of-the-art in each field. While organs-on-chip and tissue engineering are not the only medical applications of biofabrication, they serve as representative case studies for in vitro and in vivo use of biofabricated systems in medicine. The challenges faced by these subfields, and the progress made within them, can be applied to other medical applications including dynamic drug delivery and surgical planning and training.[70]

3.2.1. Organ-On-a-Chip Systems

Microfluidic devices, by allowing us to study how groups of cells coordinate their assembly and differentiation to generate functional tissues, are thus an exemplary model system for studying organs.[71] Integrated with real-time high-resolution imaging and in vitro assays, they can provide accurate and time-varying data on the genetic and metabolic activities of living cells.[72] The "organ-on-a-chip" revolution has seen significant progress in recent years, and a few highlights are outlined below.

Griffith and co-workers pioneered early advances in microfluidic biofabrication by investigating liver tissue formation and function on a chip.[73] By seeding hepatocytes in scaffolds housed within microfluidic devices, they demonstrated that controlled fluid flow could be used to replicate physiological shear stresses and oxygen supply. By replacing the initially rigid scaffold material for soft hydrogel scaffolds, Griffith and co-workers showed prolonged maintenance of viability and metabolic function in microscale liver tissues, corroborated by sustained production of albumin.[74] These biofabricated microscale liver tissues serve as platforms for understanding liver physiology and pathology, and have the potential to be used for other tissues and organ systems as well.

Similar approaches have also been employed for studying microtissue systems composed of more than one cell type. Recently, Kamm and co-workers have developed a microfluidic platform for forming and studying functional neuromuscular junctions in vitro.[75] In this system, muscle strips and optogenetic motor neurons are cultured within hydrogels in separate compartments of a microfluidic device. The outgrowth of neurites and innervation of muscle is observed, and functional synapses are confirmed via optical stimulation of the neurons leading to contraction of the muscle (**Figure 5**A).

Organ-on-a-chip systems incorporating multiple cell types have also been integrated with microfluidic devices capable of functional behaviors beyond regulation of fluid flow. For example, Ingber and co-workers have employed cyclic mechanical stretching of a flexible PDMS membrane to mimic physiological breathing cycles in a lung-on-a-chip device.[76] By culturing alveolar epithelial cells and microvascular endothelial cells on opposite sides of the membrane, they were able to replicate and study the alveolar–capillary interface, which serves as the functional base unit of the lung in vivo (Figure 5B). Ingber and coworkershave made significant strides in adapting this approach for studying functional base units in order to understand organ-level functions in a range of different human tissue types.[77]

The organ-on-a-chip systems presented above span a range of cell and tissue types, and can contribute significantly to studies of disease pathology and treatment. By mimicking in vivo systems more closely and increasing the likelihood that therapeutics tested in animal and human clinical trials, organ-on-a-chip systems can help accelerate and advance the state-of-the-art in clinical practice.

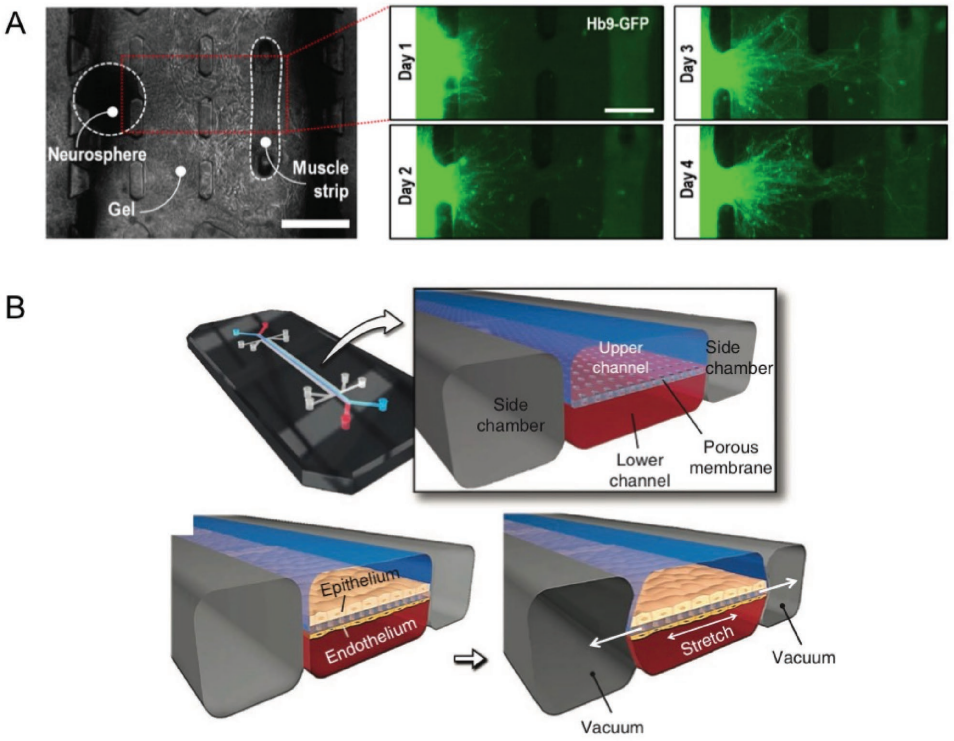

Figure 5. A) Neurospheres and muscle strips are co-cultured in a microfluidic device (left; scale bar, 500 um) and neurite outgrowth (right; scale bar, 250 um) connects the two microtissues, creating an in vitro model for neuromuscular junctions. Reproduced with permission.[75] Copyright 2015, AAAS. B) Schematic of a "lung-on-a-chip" device mimicking the alveolar-capillary interface, with physiological breathing cycles replicated by stretching the flexible membrane between the two layers of engineered tissue. Reproduced with permission.[77] Copyright 2013, Nature Publishing Group.

3.2.2. Tissue Engineering

Biofabrication of macroscale tissue and organ replacements can have a significant impact on medicine by providing patient-specific substitutes for tissue that has been damaged by disease or trauma. The primary challenge in tissue engineering is replicating the multicellular macroscale structure of biological systems, while maintaining the nano- and microscale features, mechanical properties, and adaptive functional behavior inherent to such systems.[78] Presented below are a few key demonstrations of biofabricated tissue with applications in regenerative medicine.

Several pioneering studies in engineering tissues focused on avascular tissues, such as cartilage, which do not require patterning multicellular microvascular systems throughout the tissue. Perhaps the most famous example is the "Vacanti mouse," a mouse with a human ear embedded in its back.[79] Vacanti and co-workers generated biodegradable polymer scaffolds in the shape of a human ear, seeded them with primary chondrocytes, and implanted them in immunodeficient mice. Following several weeks of implantation, the ears were harvested and shown to have retained their 3D form, and encouraged the growth of new cartilage tissue (**Figure 6**A). Recently, Spector and co-workers used 3D extrusion bioprinting to print a cell-laden hydrogel construct in the shape of a human ear, demonstrating that advanced manufacturing approaches can be used to accurately manufacture patient-specific replacements for cartilage tissue.[80]

Tissue engineering of vascularized tissue, with an ongoing challenge, has also been investigated and demonstrated in a range of tissue types. Taylor and co-workers demonstrated a seminal example of reverse engineering a macroscale vascularized organ by manufacturing a contracting heart in vitro.[81] They used perfusion of detergents to decellularize a heart, preserving its complex 3D geometry, and seeded the remaining scaffold with cardiac and endothelial cells (Figure 6B). The resulting structures were cultured within a bioreactor and demonstrated macroscopic contractions and pumping functionality mimicking cardiac function in vivo.

While replication of macroscopic vascularized structures without this type of pre-existing scaffolding can be difficult, many laboratories have demonstrated bottom-up methodologies for engineering functional subunits of vascularized tissue. For example, several approaches for engineering cardiac cell "sheets" or "patches" that can be implanted onto hearts in vivo have been demonstrated and shown to mitigate damage induced by myocardial infarction and ischemia.[82–85] Approaches for engineering other types of vascularized tissue, such as bone, kidney, and pancreas, have also met with significant success.[86–88]

Many others have cataloged the progress of tissue engineering and the advances in manufacturing technologies and novel materials that have rendered possible medical applications of biofabrication.[88–91] Rather than replicating these reviews, we will devote the next section to a discussion on how the reverse engineering of microscale and macroscale biological structures has contributed to a greater understanding of adaptive response in natural systems.

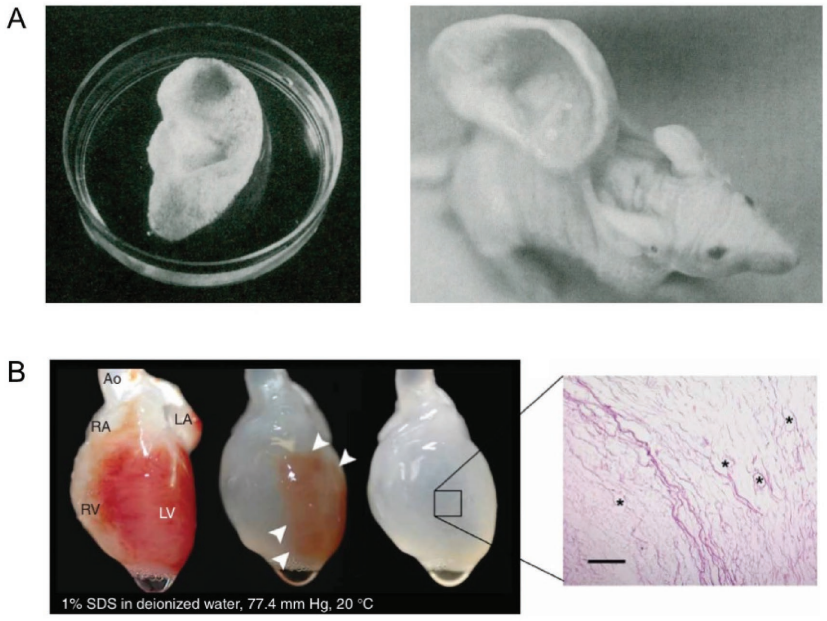

Figure 6. A) A polymer scaffold in the shape of a human ear is seeded with cartilage cells (left) and implanted subcutaneously in a nude mouse (right). Reproduced with permission.[79] Copyright 1997, LWW. B) A rat heart is perfused with a detergent to decellularize it while preserving the support structures surrounding the cells, providing a scaffold for tissue engineering a heart by re-cellularizing the scaffold. Scale bar, 50 µm. Reproduced with permission.[81] Copyright 2008, Nature Publishing Group.

3.3. Environmental Feedback and Adaptation in Engineered Biological Systems

While primary applications of biofabrication have focused on improving clinical practice, either via testing drugs in vitro or replacing diseased and damaged tissue in vivo, significant research efforts have been devoted to using biofabrication to better our understanding of biological design principles. Namely, replicating the form and function of complex biological systems has given us the opportunity to investigate the biochemical signaling cascades and intercellular communication mechanisms that drive the dynamically adaptive response we observe in nature.

Examples of engineering environmental adaptation into biological systems cover multiple tissue types. Puetzer and Bonassar have used bioreactors to recreate complex internal microarchitectures in engineered cartilage that mimic in vivo form and function.[92] By subjecting chondrocyte-laden hydrogels, injection molded in the form of a knee meniscus, to dynamic compressive loading by a structure mimicking the femoral condyle, they have shown that they can reproduce the spatial organization and composition of native tissue. Similar studies have also investigated the effect of mechanical loading on other tissue types, including skeletal muscle, which is well known to adaptively respond to "exercise" stimulation.[93] Interestingly, other forms of controlled external stimulation, such as fluid flow, biochemical signals, and electrical signals, have also been shown to have hypertrophic effects on engineered muscle tissue in vitro.[94,95] These studies showcase the ability of biofabricated tissues to serve as platforms for studying the underlying mechanisms of adaptive functional response in living systems.

Studies on adaptive response have not been limited to external stimulation that results in positive effects on engineered tissue. Indeed, biofabricated tissues serve as the ideal in vitro platform for risk-free assessment of the factors underlying tissue disease, damage, and degradation in vivo. Ingber and co-workers have, for example, used their lung-on-a-chip model to investigate how cigarette smoke affects lung physiology at the molecular, cellular, and organ levels.[96] Similar studies studying disease onset in other engineered tissues and organoids, such as skeletal muscle, brain, gut, kidney, and liver, have likewise contributed significantly to the scientific community's understanding of biological design principles.[97–99]

3.4. Remaining Challenges and Future Impact of Biofabrication

The discipline of biofabrication, while it has evolved somewhat separately from the discipline of biomimicry, has been addressing similar challenges related to both understanding and replicating biological design. Most of the advances in the early days of these fields relied on existing understanding of natural systems, as uncovered and explained by the field of biology. However, there now exists the potential for biofabrication to discover new knowledge regarding the underlying design principles that govern complex biological systems. It is therefore conceivable that, in addition to learning from the field of biology, engineers designing smart biomimetic materials will soon be drawing inspiration from the biofabricated systems discussed in this section. Moreover, translational medical applications of biofabrication require a complete understanding of the biochemical cascades and mechanical and electrical cues that govern tissue formation and function in vivo. There is thus a great need for strengthening the communication between the many subfields of biological design, as this is a prerequisite to fully replicating and taking advantage of the adaptive functionality of biological systems.

4. Biohybrid Machines and Systems

Decades of progress in biofabrication for applications in tissue engineering and regenerative medicine have had significant impact on clinical practice and outlook. Reverse engineering natural systems with the biological materials of which they are composed of have helped establish the enabling manufacturing technologies and in vitro culture practices that enable the newest subfield of biological design: biohybrid machines and systems. This subfield is motivated by the idea of forward engineering, or applying biological materials toward non-natural or hypernatural functional tasks. This approach has been applied

toward biological materials from the nano- to the macroscale, for a range of different functional behaviors and tasks.

4.1. Accomplishing Functional Behaviors in Biohybrid Systems

Some of the earliest demonstrations of biological materials are being used to power non-natural functional behaviors that used DNA as a source material for molecular machines. Neumann and co-workers, for example, used strands of DNA to construct "molecular tweezers" that used the hybridization of complementary strands of oligonucleotides to open and close.[100] Deeper understanding of the dynamics of other nanoscale biological materials, such as actin, myosin, and kinesin,[101,102] likewise spurred the creation of machines that utilized these systems as functional components. Osada and co-workers, in an effort to construct soft biological actuators for use inside the human body, used actin and myosin as components in gel machines.[103] By engineering chemically crosslinked actin filaments that moved along a gel substrate composed of crosslinked myosin, they were able to replicate the speed of actin migration in vivo at a significantly larger scale. Biological machines that use microscale biological materials, such as bacteria, protozoa, algae, and single muscle cells, have also been demonstrated and documented in other reviews.[104–106] In this progress report, we shall focus on a subset of biological machines that utilize macroscale tissue to power functional behaviors and interface with synthetic materials to form biohybrid systems. These machines that have been manufactured using both primary and engineered tissues operate at the forefront of this newest subfield of biological design.

4.1.1. Use of Primary Tissue as Biohybrid Machine Components

Cardiac muscle tissue has been one of the primary materials used to build and power biohybrid machines. Early demonstrations by Whitesides and co-workers showed that primary cardiomyocytes, derived from neonatal rat ventricles, could be used as actuators for different functional behaviors.[107] They cultured cardiomyocytes on thin PDMS films coated with extracellular matrix proteins, and used these "muscular thin films" as the fundamental building block for centimeter-scale machines of diverse geometries. The muscle was used to power rudimentary walking, swimming, and gripping in vitro (**Figure 7**A). Park and

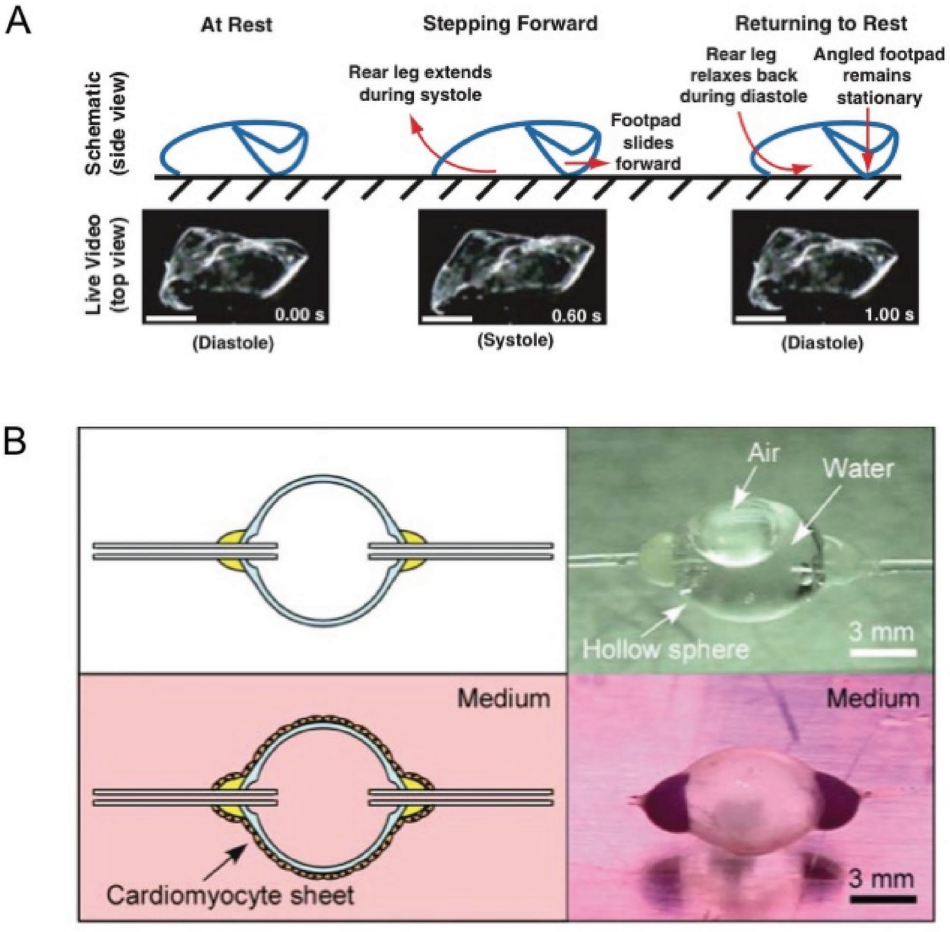

Figure 7. A) Schematic (top) and representative images (bottom) of a cardiac muscle-powered PDMS device capable of autonomous locomotion. Scale bar, 1 mm. Reproduced with permission.[107] Copyright 2007, AAAS. B) A flexible spherical pump, composed of PDMS, is actuated by a sheet of cardiomyocytes. Reproduced with permission.[109] Copyright 2007, RSC.

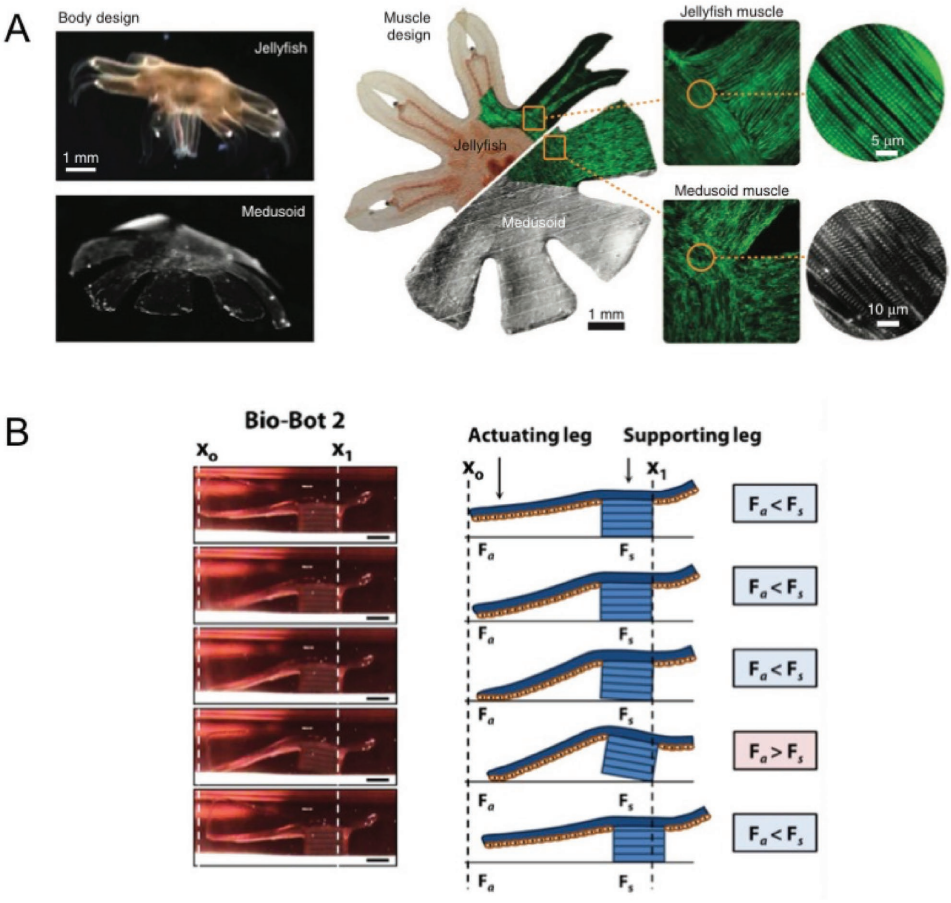

Figure 8. A) A biohybrid machine powered by cardiac muscle tissue mimics the morphology and swimming behavior of a jellyfish. Reproduced with permission.[110] Copyright 2012, Nature Publishing Group. B) A 3D-printed millimeter-scale biohybrid machine uses the autonomous contraction of cardiac muscle to crawl across a 2D substrate. Reproduced with permission.[112] Copyright 2012, Nature Publishing Group.

co-workers utilized a similar approach to engineer a walking biohybrid machine, culturing cardiomyocytes onto grooved PDMS surfaces that allowed for high-density patterning of cells onto the synthetic scaffold or "skeleton."[108] These robots, which continued to walk over a period of 10 d, demonstrated that sustained long-term functionality could be generated using such machines. Kitamori and co-workers have shown that such cardiomyocyte-PDMS composite systems can also be targeted at applications in pumping.[109] They used microfabrication approaches to engineer hollow PDMS spheres, mimicking the hollow chambers of the heart, with inlet and outlet microchannels, mimicking blood vessels (Figure 7B). Cardiomyocytes cultured around the sphere formed a contractile sheet that could deform the sphere, transporting fluid through the channels.

More recently, Parker and co-workers have shown that biological machines powered by sheets of primary cardiac cells can mimic complex functional behaviors by drawing design inspiration from natural organisms. Again relying on thin films of PDMS as a synthetic substrate for cardiac muscle sheets, Parker and co-workers mimicked the shape and muscle alignment of a jellyfish.[110] In addition to replicating the internal architecture of native cardiac muscle tissue in their biohybrid machine, they were able to replicate the stroke kinematics and propulsive swimming of real jellyfish (**Figure 8**A). Parker and co-workers applied a similar approach toward another natural organism, the stingray, to show that more complex swimming mechanisms could also be accomplished using this methodology.[111] By genetically engineering the cardiomyocytes to respond to a visible light stimulus, they were able to coordinate sequential contraction in separate regions of the biohybrid machine. This enabled biomimicry of the undulating motion of stingrays and demonstrated that visible light signals could be used to steer and the machines through an obstacle course.

While PDMS has proved to be a popular and practical substrate for manufacturing cardiac muscle-powered biological machines, it is likely that substrate materials that combine the compliance and flexibility of natural materials with their hydrophilicity and bioactivity will contribute even further to the development of biohybrid systems. We have shown that PEG-based hydrogels can serve as tunable skeletons for walking biohybrid robots powered by primary cardiomyocytes.[112] A non-natural skeleton geometry, fabricated via stereolithographic 3D printing, patterned with a sheet of cardiac cells is shown to crawl across a 2D substrate with a precisely defined "stick-slip" walking mechanism (Figure 8B).

By relying on 3D printing to manufacture the skeletons for our biohybrid machines, we were able to test a variety of different device designs and configurations for optimal functional performance. This type of iterative design and manufacturing approach is especially useful when attempting to generate functional behaviors, such as walking and swimming, in structures that do not replicate the geometry of natural organisms, such as the jellyfish and stingray case studies presented above. True forward engineered design of biohybrid systems capable of non-natural or even hypernatural functional behaviors will likely rely heavily on rapid prototyping and computational modeling to accomplish its goals.

Another tool that could greatly enhance the function and broaden potential applications of biohybrid machines is the modification of the underlying synthetic material substrate. Khademhosseini and co-workers have, for example, seeded primary cardiomyocytes onto gelatin methacrylate hydrogels with embedded carbon nanotubes.[113] These embedded nanotubes, in addition to improving the adhesion and alignment of cells on the substrate, reduced the excitation threshold for muscle contraction and helped protect the tissue from cytotoxic compounds. This type of hypernatural functional behavior could prove to be extremely useful for real-world applications of biohybrid machines.

Applications of biohybrid machines inside, or interfacing with, mammalian systems will require systems that can operate in environments that mimic the physiological temperature, pH, and humidity of mammals. The examples discussed above, for example, could all be modified to apply toward medical applications inside the human body, such as targeted drug delivery or dynamic functional implants. However, real-world applications at ambient temperature will either require on-board temperature and humidity regulation mechanisms and exoskeletons, or an alternative functional tissue system. Morishima and co-workers have demonstrated that excised insect muscle tissue can be used as an actuator to power biohybrid machines at ambient temperature.[114] These machines can function in dry environments, such as air, instead of in temperature-regulated culture medium as used in the mammalian approaches outlined above. Moreover, they can sustain this functional behavior over several days, enabling long-term applications. Morishima and co-workers have also shown that excised insect muscle can be engineered to be optogenetic and that this enables more complex functional tasks.[115] Utilizing the spatiotemporal control over muscle stimulation provided by optogenetics, they sequentially stimulated distinct regions of a biohybrid cylinder to mimic the functionality of a peristaltic pump.

Natural biological systems, in addition to generating complex actuation mechanisms and patterns, are also remarkable for their higher-level functional behaviors, such as self-assembly, self-repair, and adaptation to a wide range of environmental cues. Kaplan and co-workers have shown that insect muscle bioactuators, generated from primary stem cells, can demonstrate these types of complex coordinated responses in vitro.[116] The stem cells they use self-assembled into defined geometries and maintained functionality for several months, despite being subjected to extreme temperature and pH conditions. They also replicate in vivo-like healing behaviors in response to inflicted injury.

4.1.2. Use of Engineered Tissue as Biohybrid Machine Components

The functionality of primary cells and tissues, whether derived from mammalian or insect systems, are affected by the age and health of the animal from which they are derived. Biohybrid machines will require more reliable and sustainable sources for the biological materials which power them, motivating the need to use tissue engineered from cell lines as machine components. There has been very limited exploration of this concept in biohybrid machine design, but significant progress has been made in recent years, setting the stage for a variety of engineered tissue-powered machines for a range of functional tasks.

The cardiac muscle-based approaches described in the previous section, in addition to relying on primary cells, were further disadvantaged by the inability to provide true "on–off" control over contraction and functionality. While electrical or biochemical stimulation could be used to pace contractility in such cardiac-based systems, completely stopping and starting of contraction on-command is not a capability inherent to such systems. This is one of the primary reasons why most natural systems rely on cardiac muscle for actions that require autonomous continuous contraction, such as pumping blood, and skeletal muscle for actions that require more control, such as movement and locomotion.

We demonstrated the first examples of walking biohybrid robots, bio-bots, powered by tissue engineered skeletal muscle.[117] Using a stereolithographic 3D printer, we manufactured a flexible PEG-based hydrogel skeleton for our bio-bots, and placed them within a printed PEG-based injection mold. We then injected a liquid pregel solution, composed of skeletal myoblasts from the C2C12 cell line mixed with natural hydrogels that mimicked the extracellular matrix in vivo, into the mold. Cellular traction forces compacted the pregel solution around the printed skeleton. Transferring the resultant biohybrid structure into differentiation medium resulted in fusion of myoblasts into myotubes and development of the actin–myosin contractile apparatus (**Figure 9**A). The bioactuator was shown to generate passive tension forces comparable to other demonstrations of tissue engineered skeletal muscle.[118,119] The passive tension force generated could be increased by imposing a static mechanical stretch stimulus during differentiation, and by the addition of biochemical factors, such as human insulin-like growth factor 1 (IGF-1) to the differentiation medium. This was an important confirmation that the adaptive behavior inherent to natural biological systems could be mimicked in biohybrid systems cultured in vitro.

Since on–off control over force production is one of the primary motivations for using skeletal muscle as a bioactuator, we demonstrated that contraction could be started, stopped, and paced in a reliable and reproducible manner using electrical stimulation. We then optimized the design of the bio-bot skeleton for directional locomotion, and showed that we could regulate the direction and speed of walking in a controllable manner (Figure 9B). This design optimization required engineering geometric asymmetry into the bio-bot skeletons, which take the form of two pillars, mimicking tendons, connected by a compliant beam, mimicking an articulating joint. When stimulated electrically, the engineered skeletal muscle

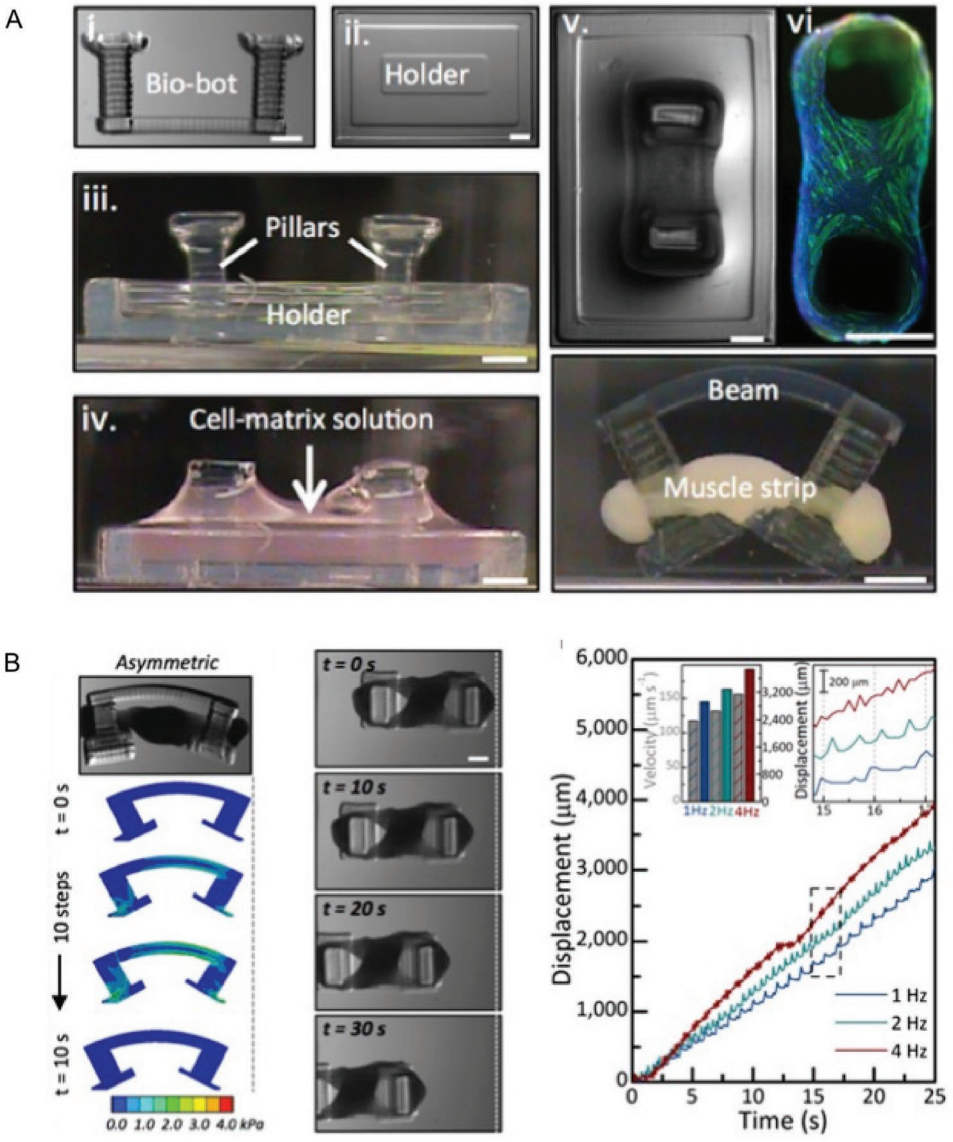

Figure 9. A) A 3D-printed hydrogel mold serves as a template for the injection and formation of tissue engineered skeletal muscle around a flexible 3D-printed hydrogel skeleton. B) An asymmetric skeleton geometry drives directional locomotion of skeletal muscle-powered biohybrid machines (left), and the speed of locomotion can be controlled by regulating the frequency of muscle electrical stimulation (right). Reproduced with permission.[117] Copyright 2014, National Academy of Science.

contracts symmetrically, but the skeleton's longer pillar deflects to a greater degree, and the bio-bot consequently crawls in the direction of the longer pillar.

Inspired by our first demonstration of engineered skeletal muscle-powered locomotion in a biohybrid machine, we used an optogenetic form of the C2C12 myoblast line to further enhance the complexity of functional behaviors that can be accomplished by such machines.[120] Taking advantage of the precise spatiotemporal control over muscle contraction enabled by optogenetics, we showed that directional locomotion and 2D rotational steering could be accomplished in completely symmetric structures (**Figure 10**A). Additionally, we showed that an "exercise" regimen of daily optical stimulation significantly enhanced muscle force production, and coupling optical and mechanical stimulation led to a further synergistic increase in muscle performance. This was the first demonstration of light-driven functional improvement in engineered skeletal muscle, and inspired our following experiments testing whether optical exercise could be used to counteract damage in our bioactuators.

Loss of muscle function, both in vivo and in our bioactuator system, is primarily driven by mechanical damage. Inspired by mechanisms of in vivo healing, we designed and optimized a protocol for healing bioactuators in vitro using targeted delivery of myoblasts and extracellular matrix proteins, sustained local release of IGF-1, and light-driven exercise. Our healing protocol drove complete recovery of force production in muscle bioactuators within 2 d postdamage.[121] This ability, to

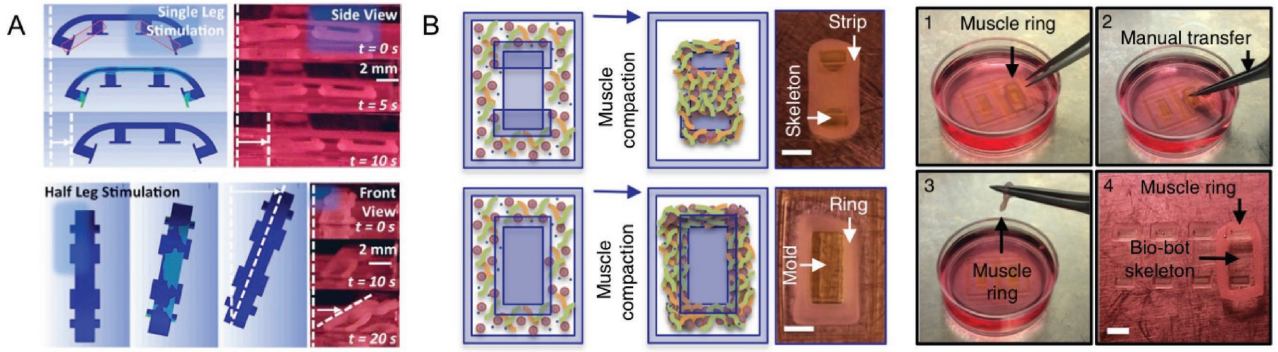

Figure 10. A) Targeted light stimulation of specific regions of an optogenetic skeletal muscle actuator in a symmetric biohybrid machine drive directional locomotion (top) and rotational steering (bottom). B) Switching from a muscle strip injection mold (top left) to a muscle ring injection mold (bottom left) allows for modular transfer of the muscle rings to any of a wide variety of skeletons (right). Reproduced with permission.[120] Copyright 2016, National Academy of Science.

dynamically adapt and respond to environmental stimuli, demonstrates the fundamental advantage of designing and building the functional components of machines using biological materials. This demonstration sets the stage for future generations of biohybrid machines which utilize principles of genetic engineering, synthetic biology, and tissue engineering to generate non-natural and hypernatural adaptive behaviors.

We chose to demonstrate "walking" as a platform example of the functionality of skeletal muscle bioactuators, but locomotion is, of course, only one of many possible applications for such biohybrid systems. To adapt our engineered optogenetic bioactuators for other applications, we have altered our manufacturing process to a two-step approach (Figure 10B). First, the muscle pregel solution is injected into a ring-shaped mold, and the resultant muscle ring is manually transferred to a bio-bot skeleton. This modular design not only allows for coupling bioactuators to a variety of different types of walking bio-bots, without modification to the muscle manufacturing process, but also enables ready adaptation to skeletons for use as pumps or conformable grippers.[122] Furthermore, the idea of modular tissue rings can be adapted for use with other cell types. We have recently shown that a ring composed of motor neurons, derived from embryonic stem cells, stacked on top of a ring composed of engineered skeletal muscle, can be used to replicate the form and function of neuromuscular junctions in vivo.[123] Chemical stimulation of the neurons can be used to control contraction of the skeletal muscle tissue, setting the stage for incorporating neural circuits into future bio-bot designs and enabling higher-level functional control and decision-making behavior in such machines.

4.2. The Ethics of Biohybrid Design

Each subfield of biological design comes with its own ethical questions and implications. Others have discussed concerns related to ethical and legal policies in this field, with a special focus on the ethics of biofabricating human tissues, stem cell origin, and gene therapy.[99,124] Ethical implications of biohybrid machine design have also been explored in the literature, though perhaps to a lesser degree due to the relative novelty of this field, and some have proposed drawing inspiration for responsible practices and policies from the field of synthetic biology.[125]

Harnessing "living materials" for application in man-made systems has the potential to excite and inspire new technologies and solutions for existing grand challenges. Scientists and engineers who conduct such research can benefit greatly from explaining the underlying motivation for this discipline and engaging the public in conversations about potential ethical implications. In collaboration with the National Science Foundation Science and Technology Center Emergent Behavior of Integrated Cellular Systems (EBICS), we have worked to spark such conversations by creating a series of vignettes centered around our bio-bots.[126] These vignettes center around a diverse set of topics, ranging from bio-bots that learn and evolve to hyperorgans that enhance human performance. Each vignette is followed by a series of questions designed to start a dialogue about the potential positive and negative impacts of such technology, as well as practices and policies that could be used to regulate such inventions.

At the center of many of these dialogues is the concept of "emergent behavior," most simply defined as a behavior demonstrated by a complex multicomponent system that is not demonstrated by its individual parts.[127,128] Until we fully understand the signaling cascades of both natural biological systems and synthetic biohybrid systems, it is arguable that we cannot fully predict the emergence of form and function in such systems. Consequently, engineering and procedural controls for forward design of biohybrid machines must be established before conducting such experiments, and must be regularly evaluated and revised. Our current experiments with walking bio-bots, for example, are conducted in regulated environments that provide us with precise control over bio-bot lifetime and performance. If we pursue applications of bio-bots in the real-world, we will need to implement fail-safes that ensure we can control lifetime and performance remotely.

Conversations about ethics, and the engineering procedures and legal policies they generate, must be an integral part of the larger conversation about biological forward design. The scientific community that creates these technologies, and the global community that is impacted by them, must work together to understand and address the ethics of biohybrid design.

4.3. Challenges and Next Steps in Biohybrid Design

In addition to the previously discussed difficulties of manufacturing multiscale multimaterial biological systems, biohybrid design faces technical challenges that are unique to this discipline. Unlike biofabrication, which centers on reverse engineering existing natural systems, biohybrid design requires forward engineering non-natural or hypernatural functionalities for which there are no existing blueprints. In addition to understanding the interactions and interfaces between the numerous and diverse components of a biological system, therefore, this requires combining these components and manipulating the communication between them in novel ways. In this report, we have presented key examples of forward engineered biohybrid machines that showcase the significant progress that has been accomplished in this field over the course of a few years. These serve as encouraging demonstrations of the future impact of biohybrid design, with initial demonstrations likely focusing on applications in healthcare, but eventually broadening to encompass and address real-world challenges in environmental regulation and national defense.

5. The Future of Biological Design

We have presented the motivation, development, and potential impact of three of the predominant subfields of biological design, and discussed how progress in the two more established subfields of biomimicry and biofabrication has converged to create and advance the emergent subfield of biohybrid systems design. This convergence is not a one-way street, but rather the creation of an interconnected network that encourages continuous feedback between the subfields of biological design. There has never been a greater need for interdisciplinary communication and connectivity, as each subfield will contribute important fundamental knowledge regarding the design principles that govern adaptive systems, and this knowledge will be applicable to the discipline as a whole.

An important requirement that remains in biological design is a robust and reliable educational system for training the next generation of engineers and scientists to address ongoing challenges and future applications of this technology. Thus far, there has been very limited investigation and development of novel educational models for biological design, and most studies have discussed this in the context of the broader discipline of bioengineering.[129,130] We have attempted to bridge this gap by developing a problem-based course at the interface of biofabrication and biohybrid design, targeted at an undergraduate engineering audience.[131] In addition to teaching students the practical use and limitations of enabling technologies for this field, such as 3D printing, this course trains students to solve ill-structured technical problems in relatively unexplored scientific fields, such as biohybrid machine design. Formal evaluation of this educational model has shown that students benefited greatly from formulating and testing their own hypotheses in a laboratory setting, and demonstrated enhanced and sustained interest in biological design after the conclusion of the semester-long course.

In addition to training the next generation of engineers and scientists to address the technical challenges of biological design, we must also train them, as well as policy makers, to engage in productive and impactful discourse on the ethical implications of this work. Recent discussions of bioengineering ethics have emphasized the need for continually re-evaluating engineering practices, and educating engineers to actively engage in and respond to new policies.[132,133] This is equally important, if not even more so, in the emergent discipline of biohybrid design, as a consequence of its rapid evolution and expanding impact over recent years.

Developing new pedagogical techniques for training innovators in biological design is not the only significant challenge facing this field. There are practical challenges associated with the large-scale production, transport, and storage of biohybrid systems to which very few robust solutions exist. Introducing such systems to the global economy will thus require significant advancements and contributions from stakeholders in biomanufacturing, cryopreservation, supply chain management, and international trade. Once solutions to these multifaceted challenges are found and optimized, biological design can truly be incorporated into the "toolbox" of every engineer.

Dynamic problems require adaptive solutions, and biological design has given us the materials and manufacturing processes required to engineer systems that sense and respond to their environment. By developing new educational models for teaching biological design, and integrating ethics and policy discussions in every stage of biological design, we can develop a holistic approach for addressing societal challenges with biomimetic, biofabricated, and biohybrid solutions. Biological systems have taught us that the most complex functional tasks can be accomplished via adaptation and evolution, and we too must learn to adapt and evolve to an ever-changing scientific landscape.

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Conflict of Interest

The authors declare no conflict of interest.

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