BIOMATERIALS

Biohybrid actuators for robotics: A review of devices actuated by living cells

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Actuation is essential for artificial machines to interact with their surrounding environment and to accomplish the functions for which they are designed. Over the past few decades, there has been considerable progress in developing new actuation technologies. However, controlled motion still represents a considerable bottleneck for many applications and hampers the development of advanced robots, especially at small length scales. Nature has solved this problem using molecular motors that, through living cells, are assembled into multiscale ensembles with integrated control systems. These systems can scale force production from piconewtons up to kilonewtons. By leveraging the performance of living cells and tissues and directly interfacing them with artificial components, it should be possible to exploit the intricacy and metabolic efficiency of biological actuation within artificial machines. We provide a survey of important advances in this biohybrid actuation paradigm.

INTRODUCTION

To efficiently tackle the challenges of today's world, robotics is currently undergoing a deep revolution in both its design principles and constitutive elements. New robotic paradigms are rapidly emerging at different scales. At the macroscale, soft robots are expected to lead to the development of squeezing, stretching, climbing, growing, and morphing machines (*1*) that are intrinsically safe and can act as cooperative systems in many applications (*2*). At the microscale, the development of new fabrication techniques and the miniaturization of electronic controllers and sensors have opened the design space to make controllable robots as small as insects or even submillimeter devices (*3*).

In each of these research areas, actuation is a critical aspect. Traditional and recently developed actuation technologies not only offer a wide range of opportunities but also have clear limitations. For example, electromagnetic motors are used in a large set of machines, but they are heavy, do not guarantee a high energy density, and make it difficult to design large contractile strokes with a small footprint (*4*). Other technologies have different drawbacks; for example, pneumatic and hydraulic actuators require a cumbersome activation system, whereas some electroactive polymers (EAPs) require very high driving voltages.

Animal motion is driven by coordinated movements of molecular motors only a few nanometers in size, which are hierarchically organized to produce macroscopic contractile muscle tissues. The combination of (i) individual muscle cells with intrinsic molecular machinery to regulate contraction and (ii) neural innervation of these muscle cells in three dimensions (3D) provides a scalable control scheme for modulation of force and deformation. Most of the performance characteristics of muscles are difficult to replicate using current synthetic actuators. Further, muscles control their stiffness by recruiting a variable number of myofibers; they are highly scalable (ranging in mass from a few micrograms in small insects to several hundred kilograms in whales), self-healing, and eco-compatible.

Although variable stiffness actuators can be produced in electric motors using combinations of impedance control, inherent compliance, and damping or inertial systems (*5*), many other key properties of natural muscles cannot be matched (*6*). For example, although piezoelectric motors can be miniaturized (*7*), they have a very limited stroke, and they need (as do all artificial motors) to be powered by batteries, which cannot be efficiently scaled down in size at present. Most other traditional actuators cannot be scaled down to the microscale without losing force production and controllability. Molecular motors based on DNA, proteins, or synthetic molecules are a fascinating option (*8*), but their success has been limited, because they are characterized by very poor forces/torques and are challenging for system integration, easy control, and upscaling.

This means that current machines (even soft and collaborative ones) are not able to replicate many lifelike movements typical of animals (*6*) and highlights the need for high-performance, flexible actuators able to recapitulate or even outperform natural muscle functions. One potential solution to achieve efficient actuation of minidevices (larger than 1 mm) and microdevices (below 1 mm) is to exploit biological systems to construct biohybrid robots.

Biohybrid robotics represents an exciting paradigm based on the integration of properly engineered artificial structures and living biosystems. This approach exploits the unique characteristics of biological cells and tissues, which have been refined over millions of years of natural evolution. Actuation is a key function and constitutes the main motivation for the development of biohybrid robots (*9*). Biohybrid actuators have the potential to provide artificial devices with unprecedented performances at the macroscale and to enable the development of self-powered microrobots. The growing interest in this research field is demonstrated by the bibliometric analysis in Fig. 1.

Figure 1A demonstrates that, until 2011, the field of biohybrid actuation was mainly characterized by exploratory conference papers rather than journal publications. Starting from 2012, the pool of journal papers published on this topic has been growing exponentially.

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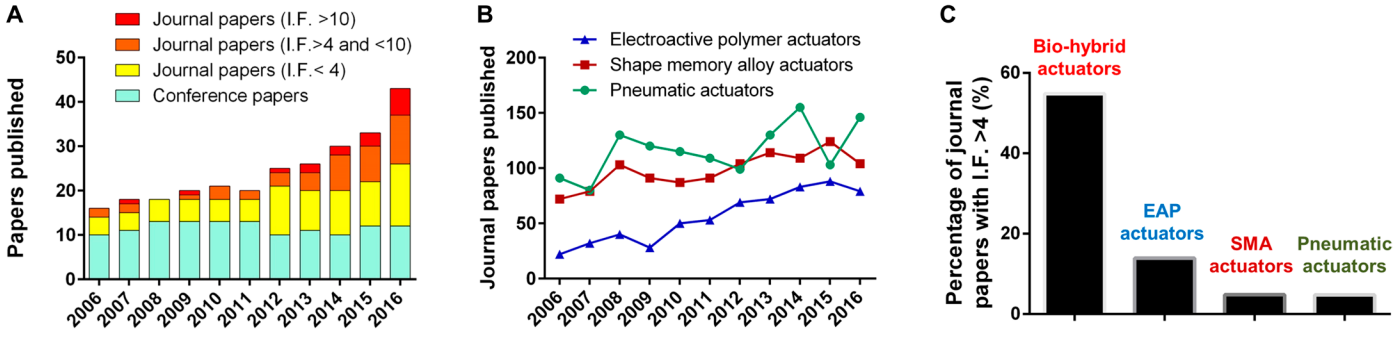

Fig. 1. Bibliometric analysis of the biohybrid actuation field and few competing ones. (**A**) Papers published from 2006 to 2016 on biohybrid actuators divided in conference papers, journal papers with I.F. <4, journal papers with I.F. between 4 and 10, and journal papers with I.F. >10. The analysis was conducted on the Scopus database by using the following keywords in the title and abstract: "bio-hybrid actuator," "biohybrid actuator," "cell-based actuator," "bioactuation," "bacteria robot," "muscle cell actuator," "muscle-based actuator," "biohybrid robot," "bio-hybrid robot," "biohybrid system," "bio-hybrid system," "biohybrid device," and "bio-hybrid device" (from this list, only the papers closely dealing with bioactuation were then selected manually). (**B**) Journal papers published from 2006 to 2016 on smart materials–based and pneumatic actuators. The analysis was conducted on the Scopus database by using three different keywords (in title and abstract) for the three categories analyzed: "pneumatic actuator," "shape memory alloy actuator," and "electroactive polymer actuator." (**C**) Percentage of journal papers with I.F. >4 with respect to the overall number of journal papers published in 2016 on biohybrid actuators, EAP, SMA, and pneumatic actuators.

Although the momentum generated by this research field is still relatively small, it is rapidly building up with a series of high-impact results. Comparing the trend of journal papers published on biohybrid actuation (Fig. 1A) with those featuring pneumatic actuators, shape memory alloy (SMA) actuators, and EAP actuators (Fig. 1B), it is clear that biohybrid actuation is growing more quickly. Actuators based on pneumatic and smart materials–based principles derive from more consolidated technologies and are thus featured in a larger number of applications (explaining the larger number of papers published), but they have less potential to generate new basic knowledge. This is confirmed in Fig. 1C: The percentage of journal papers with impact factor (I.F.) >4 relative to the overall number of journal papers published in 2016 is very high for biohybrid actuation (55%) but is notably lower for EAP actuators (14%), SMA actuators (5%), and pneumatic actuators (5%). This further suggests that the biohybrid actuation research is driven by highly ambitious fundamental scientific hypotheses rather than novel applications.

Recent review papers have described the different types of mini- and microdevices actuated by living cells (*9*–*12*). Some review papers also highlighted the possible evolution of this field in terms of heterotypic (multicellular) and multifunctional biohybrid systems (*13*, *14*) and the integration and maintenance of living components for building more sophisticated soft robots (*15*). However, a quantitative evaluation of the state of the art in biohybrid actuation, targeted at the robotics-oriented audience, is not currently available.

We aim to provide an exhaustive overview of biohybrid actuators for robots at different scales proposed in the scientific literature and to analyze their current performances, comparing them with fully artificial counterparts and identifying the open challenges and possible real-world applications. We hope that this overview will drive efforts toward the development of long-term functional biohybrid machines, which will be likely grounded on a strongly interdisciplinary research framework. We chose to divide biohybrid actuators in two macro categories: (i) application-oriented nonscalable ones and (ii) general-purpose scalable ones. The first category is grounded on fully integrated biological systems to be taken and used as they are, with no or minimal modifications. This can be considered as a top-down approach, in which artificial technologies are built around the already optimized biological system that is not scalable in dimensions. The highly specialized nature of such biological elements makes this kind of biohybrid actuators suitable for specific applications, rather than general-purpose tasks. The second category deals with biological components (i.e., cells) that can be engineered and assembled according to the desired system shape and function by pursuing a bottom-up approach. Thus, they can be considered general-purpose systems that can be scaled up or down in a variety of dimensions.

APPLICATION-ORIENTED AND NONSCALABLE BIOHYBRID ACTUATORS

Figure 2 shows two classes of biohybrid actuators, based on whole "mature" biological systems, harnessed to provide artificial devices with motility. Such classes deal with systems at the microscale (motile bacteria or other microorganisms and motile cells; Fig. 2A) and at the macroscale (explanted whole-muscle tissues; Fig. 2B). In both cases, the living elements cannot be scaled down or up in size but exploited only in their original form. This also implies that such bioactuators cannot be used for general purposes but are suitable for specific contexts (targeted drug delivery or lab on a chip in the case of microscale motile cells and swimming robots or fluidic pumps in the case of whole-muscle tissues), thus highlighting the application-driven nature of these actuation solutions.

Actuators based on bacteria and other motile cells

Some microorganisms show intriguing properties, such as high-speed motility (with speeds up to more than 100 body lengths per second), which have made them very attractive for microrobotics (*10*). Biohybrid robots falling into this category (Fig. 2A) are distinguished by small dimensions (typically 1 to 3 μm) and are thus compatible with the dimensions of human capillary networks and interstitial spaces. This characteristic has fostered the application of these robots in the vascular network as drug delivery systems (*16*, *17*). These robots are also characterized by taxis response, namely, the ability to autonomously move toward a specific environmental stimulus: chemical gradients in the case of chemotaxis, magnetic fields in the case of magnetotaxis, electric fields in the case of galvanotaxis, light in the case of phototaxis,

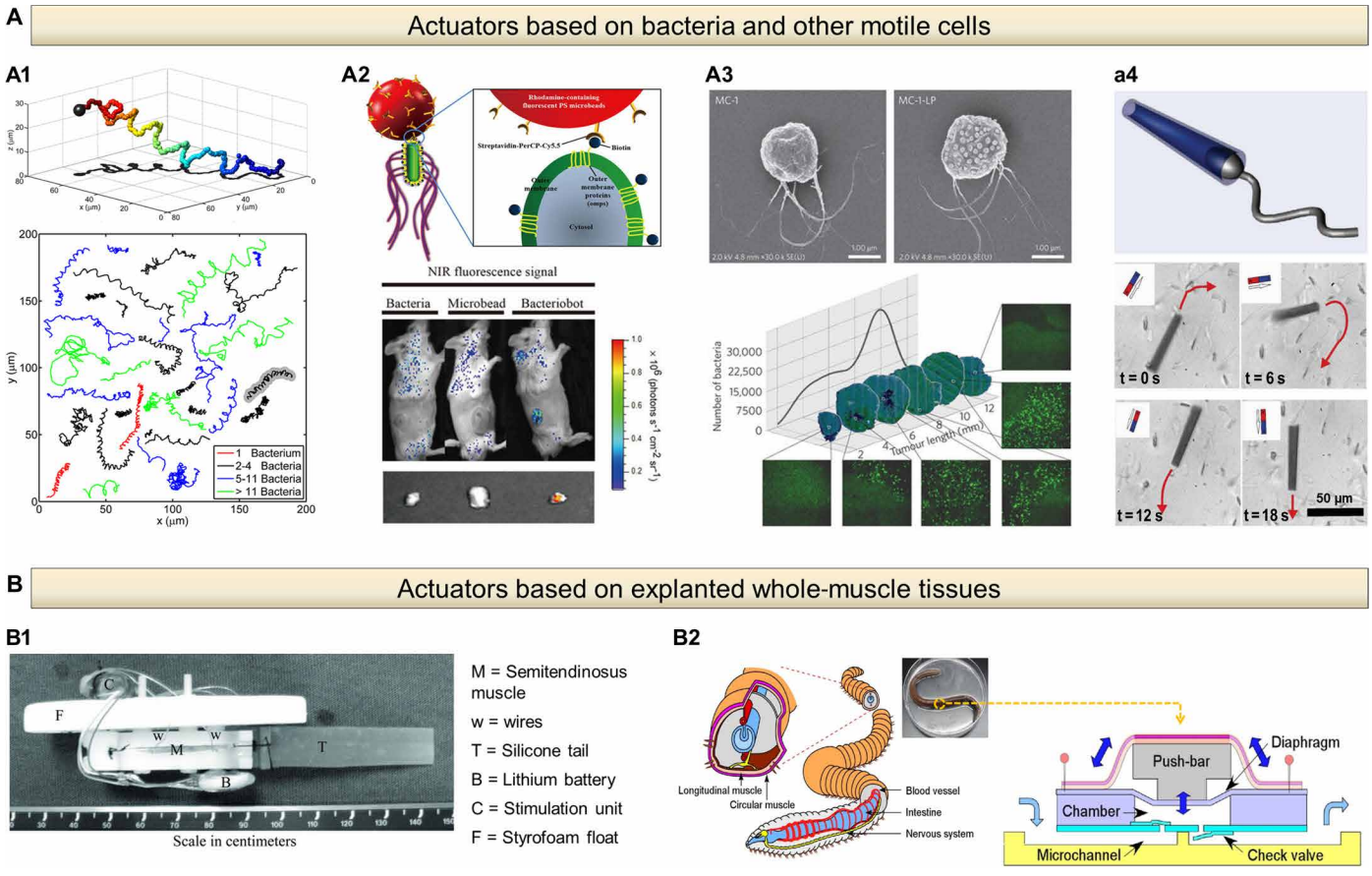

Fig. 2. Examples of application-oriented and nonscalable biohybrid actuators. (**A**) Actuators based on bacteria and other motile cells. (A1) Typical 3D (top) and 2D (bottom) trajectories for bacteria-driven beads (*19*). Reprinted from (*19*) with permission from AIP Publishing. (A2) Bacteriobot based on *S. typhimurium* (top) and its biodistribution in a tumor-bearing mouse (bottom) (*21*). Reprinted from (*21*) with permission from Macmillan Publishers Ltd. (A3) Scanning electron microscopy images of unloaded (top left) and liposome-loaded (top right) motile *M. marinus* and transverse tumor sections of the liposome-loaded microorganism after targeting (bottom) (*26*). Reprinted from (*26*) with permission from Macmillan Publishers Ltd. (A4) Representation of a motile sperm cell within a magnetic microtube (top) and its magnetically controlled locomotion at different time points (bottom) (*28*). (**B**) Actuators based on explanted whole-muscle tissue. (B1) Image of a swimming robot powered by explanted frog muscles (*29*). Reprinted from (*29*) with permission from Wiley. (B2) Fluidic pump powered by an earthworm muscle (*30*). Reprinted from (*30*) with permission from Elsevier.

temperature in the case of thermotaxis, and oxygen concentration in the case of aerotaxis. Such a variety of behaviors does not allow full control of these robots but enables sensing of the environment and autonomous steering toward preprogrammed environmental stimuli (*18*).

Research efforts have been focused on modeling and optimizing the motion of bacteria-based microswimmers in the presence of a cargo (Fig. 2A1) (*19*) and a chemotactic stimulus (*20*). Recent examples of microorganism-based biohybrid robots have demonstrated the clinical potential of such devices: Park and colleagues (*21*) proposed a "bacteriobot" based on attenuated *Salmonella typhimurium* attached to a polystyrene microbead and provided with chemotactic ability toward tumor spheroids. The authors demonstrated the theranostic ability of such system, which could target solid tumors in mice. In another study, *Escherichia coli* was coupled to a soft double-micelle microemulsion for active transport and delivery of cargo (imaging agents, genes, and drugs) to cells cultured in vitro (*22*).

In general, the potential of bacterial strains as tumor-targeting vectors has been widely highlighted (*23*, *24*). However, such "nonrobotic" strategies may improve drug targeting but remain rather far from being optimal. In addition, this drug delivery is normally systemic, raising issues of systemic toxicity and therapeutic index reduction.

Such drawbacks can be mitigated through nonsystemic delivery and robotic control approaches, for example, with the use of magnetic field–based steering followed by a taxis. Park *et al.* (*25*) proposed a microswimmer for targeted active drug delivery, where *E. coli* was attached to the surface of drug-loaded polyelectrolyte multilayer microparticles with embedded magnetic nanoparticles. Such microswimmer had chemotactic ability toward tumor tissues and could be steered remotely using external magnetic fields. Felfoul *et al.* (*26*) described a biohybrid robot based on a magnetotactic bacterium (*Magnetococcus marinus*), loaded with liposomes containing an anticancer drug. The system was both magnetotactic and aerotactic. The aerotaxis-based self-steering, together with remote magnetic steering, allowed efficient targeting of colorectal xenografts in mice and penetration to the internal (hypoxic) regions of the tumor.

This type of device cannot be scaled into larger dimensions, because the size of the biological unit is fixed; these systems operate at the microscale. Pathogenicity or immunogenicity of bacterial strains is often claimed as one of the main limitations of these systems. However, nonpathogenic forms can be found (or engineered), thus mitigating such risk. In addition, alternative motile cells have been proposed, including macrophages (*27*) and sperm cells embedded within magnetic

microtubes (*28*). Movie S1 shows some examples of biohybrid robots in the category described in this section.

Actuators based on explanted whole-muscle tissues

To increase the size of biohybrid systems, larger biological actuation units are needed. Muscle is the best candidate to this purpose. One of the pioneering studies using living muscles to power macroscopic robots (Fig. 2B) dates back 2004: Herr and Dennis (*29*) developed a swimming device powered by two explanted frog semitendinosus muscles activated through an embedded microcontroller (Fig. 2B1). The robot successfully performed simple maneuvers and straight-line swimming with a speed of $^1/_3$ body length per second. Although exciting, this approach was abandoned in the following years. Using explanted muscle tissues implies several drawbacks, such as the need to sacrifice animals, limitations in choosing actuator dimensions and architectures (depending on available animal muscles), and poor stability out of the body (the lifetime of the mentioned robot was ~42 hours). After more than a decade, few papers in the category of explanted whole-muscle tissue–based systems have been published. Tanaka *et al.* (*30*) used the earthworm skeletal muscle to power a micropump (Fig. 2B2). The bioactuator was able to generate a contractile force of 9.33 mN, driving a pump flow rate of 5.0 μl/min. Other biohybrid devices were recently fabricated by excising *Aplysia californica* muscles (*31*) and neuromuscular tissues (*32*). Although notable for providing longer lifetime and reduced ethical issues in comparison with mammalian-explanted muscles, these bioactuator types provided limited flexibility in customizing robot shapes and dimensions. Movie S2 shows the activity of biohybrid actuators based on explanted muscle tissues.

GENERAL-PURPOSE AND SCALABLE BIOHYBRID ACTUATORS

A top-down approach (based on microorganisms or explanted mature muscles) limits the possible biohybrid actuator types to preexisting living systems. The need for enhanced flexibility in building biohybrid robots adequate to be scaled up or down is better achieved using a bottom-up approach, based on single cells assembled together to form defined robot architectures. The following sections describe the different categories of biohybrid actuators based on a bottom-up approach, aiming at general-purpose and highly scalable actuation solutions.

Actuators based on cardiomyocytes

Cardiomyocytes are striated muscle cells that, when grown in contact with each other, couple to form an electrically integrated syncytium and contract as a coordinated unit. This requires tissue-engineering approaches and has led to the progressive colonization of the biohybrid robotics field by groups whose main research interests are focused on regenerative medicine and tissue regeneration technologies.

Primary cardiomyocytes obtained from neonatal rat or mouse hearts have been widely used because these cells retain sufficient developmental plasticity to be seeded onto artificial substrates and form integrated devices (Fig. 3A). A seminal study by Xi *et al.* (*33*) in 2005 described cardiomyocytes seeded on a Si-based micromechanical structure (Fig. 3A1). The resulting walking biohybrid microrobot reached a speed of 38 μm/s, equal to ~$^1/_4$ body length per second. Tanaka *et al.* (*34*) successfully used the same cell type to power a micropump (Fig. 3A2), obtaining a microchannel flow rate of 2 nl/min. Although the performance using cardiomyocytes is much less than the micropump powered by earthworm muscles, the high design flexibility and system dimensional scalability are major advantages. For example, a larger pumping device using cardiomyocytes was described by Park *et al.* (*35*).

More complex cardiomyocyte-powered soft robots have been developed in the following years. In 2007, a swimming crab-like biohybrid robot based on cardiomyocytes coupled to a polydimethylsiloxane (PDMS) structure was proposed (*36*). The robot had an average velocity of 100 μm/s and traveled ~50 m over a 1-week period. In the same year, Feinberg *et al.* (*37*) introduced a series of highly customized biohybrid microrobots based on patterning-aligned monolayers of cardiomyocytes on PDMS thin films that could be easily formed into multiple devices (Fig. 3A3). In this way, the authors achieved centimeter-scale constructs able to grip, pump, walk, and swim, generating stresses up to ~4 mN/mm^2. The evolution of this work was published in 2012, where the body shape and muscle architecture of a jellyfish was mimicked through proper cell patterning to engineer a biohybrid robot that could swim using the same motion as the real animal (Fig. 3A4) (*38*). Specifically, a computer-aided design approach was used to effectively reproduce the power and recovery phases.

Advances in biomaterials and tissue-engineering methods have the potential to further improve cardiomyocyte-powered biohybrid robotics. New materials are being developed to improve cardiomyocyte function, such as the integration of conductive carbon nanotubes (CNTs) in gelatin methacrylate hydrogels (Fig. 3A5) (*39*, *40*). These CNTs, besides providing local electrical triggering, improved cardiac cell adhesion, organization, and cell-cell coupling compared to hydrogels without CNTs, but these and other engineered cardiac tissues still function poorly as compared with native cardiac muscle tissue. Yoon *et al.* (*41*) fabricated poly(D,L-lactide-co-glycolide) microcylinders and coupled them with cardiac cells, obtaining a substantial contraction of these structures (Fig. 3A6). In addition to materials engineered to sustain cell viability and functions, research efforts are also focusing on advanced fabrication techniques to increase biohybrid system performance. Chan *et al.* (*42*) developed miniaturized "biobots" from poly(ethylene glycol) diacrylate hydrogels and cardiomyocytes using a 3D printer, obtaining a walking speed of ~236 μm/s. Last, cardiomyocytes have also been used to build devices similar to microorganism-powered swimming microrobots, although with larger dimensions. Williams *et al.* (*43*) reported a long PDMS filament provided with a cardiomyocyte on its tail, which allowed robot propulsion at 81 μm/s, thus partly mimicking flagella behavior at low Reynolds number (Fig. 3A7).

Actuators based on optogenetically modified cardiomyocytes

Although cardiomyocytes can generate large forces (that allow them to actuate deformable structures), their potential is limited by their self-contractile nature, which makes them difficult to control by an external user. Because controllability is a primary requirement for any robot design, recent efforts have focused on the regulation of contraction. One exciting approach is to use optogenetics: using microbial agents or viruses to express light-responsive proteins in living cells and therefore control various processes with externally applied light. The potential of optogenetics is well known, especially in the field of neuroscience (*44*).

The same paradigm can be easily applied to muscle cells. In 2016, Park *et al.* (*45*) developed a biohybrid device inspired by the ray (Fig. 3B) by patterning cardiomyocytes onto a four-layer elastomeric/metal structure. Cardiac cells were optogenetically modified to express a light-sensitive ion channel, channelrhodopsin-2 (ChR2). This made the cells sensitive to blue light at powers of ~10 mW. The cardiac syncytia

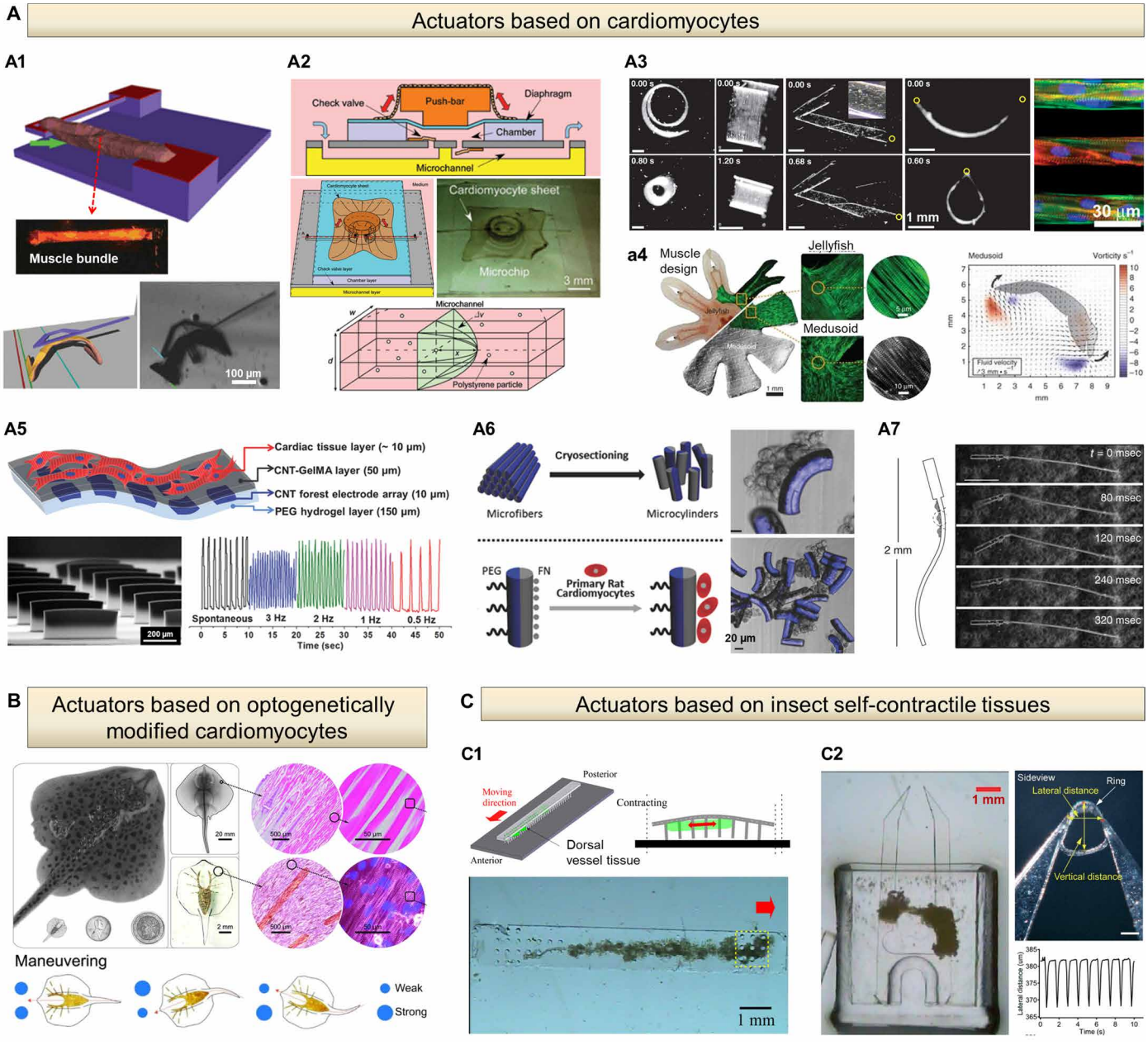

Fig. 3. Examples of biohybrid actuators based on mammalian and insect self-contractile cells/tissues. (**A**) Actuators based on cardiomyocytes. (A1) Muscle-powered microdevice based on a muscle bundle self-assembled across two anchors (*33*). Reproduced from Xi *et al.* (*33*) with permission from Macmillan Publishers Ltd. (A2) Concept and prototype of a bioactuated pump (*34*). Reprinted from (*34*) with permission from the Royal Society of Chemistry. (A3) Thin elastomeric films with customized functionalities contracted by properly aligned cardiomyocytes (*37*). Reprinted from (*37*) with permission from AAAS. (A4) Jellyfish 2D muscle architecture and reverse-engineered medusoid (left), supported by computer simulation (right) (*38*). Reprinted from (*38*) with permission from Macmillan Publishers Ltd. (A5) Multilayer hydrogel sheet impregnated with aligned CNT microelectrodes and seeded with cardiomyocytes (*40*). Reprinted from (*35*) with permission from Wiley. (A6) Microcylinders contracted by cardiomyocytes seeded on the non-PEGylated side of the microstructures (*41*). Reprinted from (*41*) with permission from Wiley. (A7) Biohybrid swimmer powered by cardiomyocytes cultured on its tail (*43*). Reprinted from (*43*) with permission from Macmillan Publishers Ltd. (**B**) Ray fish–mimicking soft robot powered by optogenetically modified cardiomyocytes and controlled through blue light pulses (*45*). Reprinted from (*45*) with permission from AAAS. (**C**) Actuators based on insect self-contractile tissues. (C1) Walking robot powered by insect DVT and operable at room temperature (*47*). (C2) Microgripper actuated by insect DVT (*48*). Reprinted from (*48*) with permission from the Royal Society of Chemistry.

on the construct could propel the device by producing forward thrust via the undulatory motion of its fins. Speed and direction were controlled by modulating the frequency of light stimuli and by synchronously or asynchronously triggering right and left serpentine circuits embedded in the device. The ray could be guided along curved paths by alternating forward motion and turning maneuvers. The biohybrid robot had an average speed of ~1.5 mm/s and could travel continuously for distances up to ~250 mm, 15 times longer than its body length. The lifetime of the system was 6 days (during which the speed remained above 80% of the initial one).

This paradigm is exciting, because it couples the high contraction force of cardiomyocytes to a degree of controllability enabled by optogenetics. However, the robot controllability by an external user is limited: Light stimuli only allow the speed or the direction of motion to be modulated but cannot switch robot activity on and off. Although for some applications this can be considered a minor issue, it needs to be taken into account when pursuing a fully controllable biohybrid robot. Optogenetics has the potential to overcome this issue: Ion channels can be modified to hyperpolarize excitable cells so that they cannot fire an action potential without optical stimulation. This would enable the use of light to turn the system on and off. Movie S3 shows different biohybrid platforms based on cardiomyocytes, including optogenetically modified ones.

Actuators based on insect self-contractile tissues

Insect tissues have evolved to survive a broader range of external and internal conditions than those of mammals, birds, or even larger vertebrate ectotherms. Despite their small body size, most insects regulate their internal environment, but when they cannot, they often tolerate wide-ranging internal fluctuations in temperature, osmolarity, oxygen availability, and pH. Small body size and extraordinary taxonomic diversity have provided an extraordinary range of solutions to environmental challenges over evolutionary time. Insects live and locomote in temperatures well below 0° to nearly 55°C, external pH from 4 to 11 in osmolarities over a wide and sometimes rapidly varying range, and oxygen pressures one-fifth of the value that would cause a human extreme respiratory distress and can survive and recover from exposure to total hypoxia for up to days. They display normal behavior during and after radiation dosages orders of magnitude higher than those that would rapidly incapacitate and kill a human.

Consequently, actuators based on insect self-contractile tissues have been proposed as an intriguing solution to enhance the robustness and usability of biohybrid devices (Fig. 3C). The excision and culture of self-contractile dorsal vessel tissues (DVTs) (corresponding to mammalian cardiac tissues) was established and optimized for lepidopteran species, such as the *Ctenoplusia agnata*. This enabled, in 2009, pillar-deflecting bioactuators to function at room temperature without medium replacement for 90 days (*46*). Afterward, biohybrid autonomous robots based on such tissues were proposed: A polypod microrobot powered by inchworm DVT was reported in 2012 (Fig. 3C1). The system walked at a speed of 3.5 μm/s and operated at room temperature (*47*). In 2013, another room temperature functional device was developed by coupling the DVT with microtweezers and packaging the whole system within a small capsule filled with culture medium (Fig. 3C2). This architecture allowed the authors to use the biohybrid system in air, out of the incubator, for more than 5 days (*48*). Although the ability to work at room temperature is not unique to the insect muscle (mammalian cells can also work in these conditions), insect cells can guarantee higher stability for significantly longer periods of time.

Using insect cells/tissues instead of mammalian ones has the potential to create very robust bioactuators. However, the mentioned prototypes were all affected by low controllability of the system, which relied on the autonomous contractile nature of the tissues itself. Although recent efforts have focused on systematically investigating different stimulation approaches for controlling insect cells (*49*), this remains the main issue to be addressed for this type of devices. Movie S4 collects some examples of biohybrid systems based on insect self-contractile tissues.

Actuators based on engineered skeletal muscle tissue

Skeletal muscle is the bioactuator of choice for biohybrid machines that require complete on-off controllability and modular design. More than 600 skeletal muscles in the body, linked to bones and controlled by motor neurons, allow humans to perform dynamic tasks with precise control. These muscles are modular—they are composed of parallel bundles of fibers formed from the fusion of precursor myoblasts into multinucleated contractile myotubes. In vivo, action potentials from motor neurons open voltage-gated ion channels in myotube membranes. An influx of calcium ions through these open channels activates actin-myosin contractile machinery, switching the myotube from a resting state (off) to a contracted state (on).

Engineering mature skeletal muscle fibers starting from myoblasts is a challenging task, and most efforts in this field have focused on extracting muscle precursor cells or excising mature muscle fibers from in vivo tissue. Forming contractile fibers in vitro from a myoblast cell line is an ongoing challenge, and as a result, this subfield is strongly tied to tissue engineering.

One of the first attempts to produce engineered skeletal muscle for potential use in biohybrid robots was undertaken by Dennis and Kosnik (*50*) (Fig. 4A). The authors generated a 3D skeletal muscle tissue construct (myooid) from primary rat myogenic precursor cells and achieved contraction forces of 3 to 30 μN (about 1% of the contraction force of a rat adult skeletal muscle). Despite these encouraging results, this approach was almost abandoned for three main reasons: (i) The engineering process was inefficient, and the construct remained arrested in an early developmental state due to the absence of signals able to promote the expression of adult myosin isoforms; (ii) the construct was limited by fixed dimensions—it did not have a vascular network and could not be scaled up in size due to nutrient diffusion constraints; and (iii) the myooid design was not readily adaptable to custom robot shapes and dimensions.

Because many of these limitations resulted from the 3D structure of these constructs, most subsequent efforts focused on engineering 2D constructs by seeding and maturing cells on flexible artificial substrates. These substrates could be either a robotic skeleton or simple cantilevers or bridges designed to assess the differentiation and contractile dynamics of engineered skeletal muscle (Fig. 4B) (*51*–*55*). Inherent structural rigidity hampered macroscale contraction in most of these systems. Starting in 2010, the mechanical properties of materials on which cells were seeded started to attract more profound attention. This drove important efforts to optimize biointerfaces/nonbiointerfaces and enhance system-level actuation.

In 2010, Kaji and colleagues (*56*) proposed a microporous alumina membrane coated with an atelocollagen layer showing muscle-like stiffness. The authors demonstrated that continuous electrical stimulation of these 2D constructs increased the formation of stimuli-responsive mature myotubes. In the same year, Ricotti *et al.* (*57*) explored ultrathin polylactic acid films for skeletal muscle engineering (Fig. 4C1). Although no contraction experiments were reported, the ultralow thickness of these systems (~320 nm) and the consequent high flexibility made them promising for biohybrid actuation purposes. In 2011, Nagamine and colleagues (*58*) proposed a patterned fibrin gel sheet with embedded electrodes (made of an electrically conductive polymer) (Fig. 4C2). They achieved on-demand muscle contraction, although the contraction values (~0.2%) were significantly lower than the ones typically observed in vivo. Another engineered hydrogel system for 2D skeletal muscle culture, based on methacrylated gelatin, was fabricated by Hosseini *et al.* (*59*). In this study, the authors

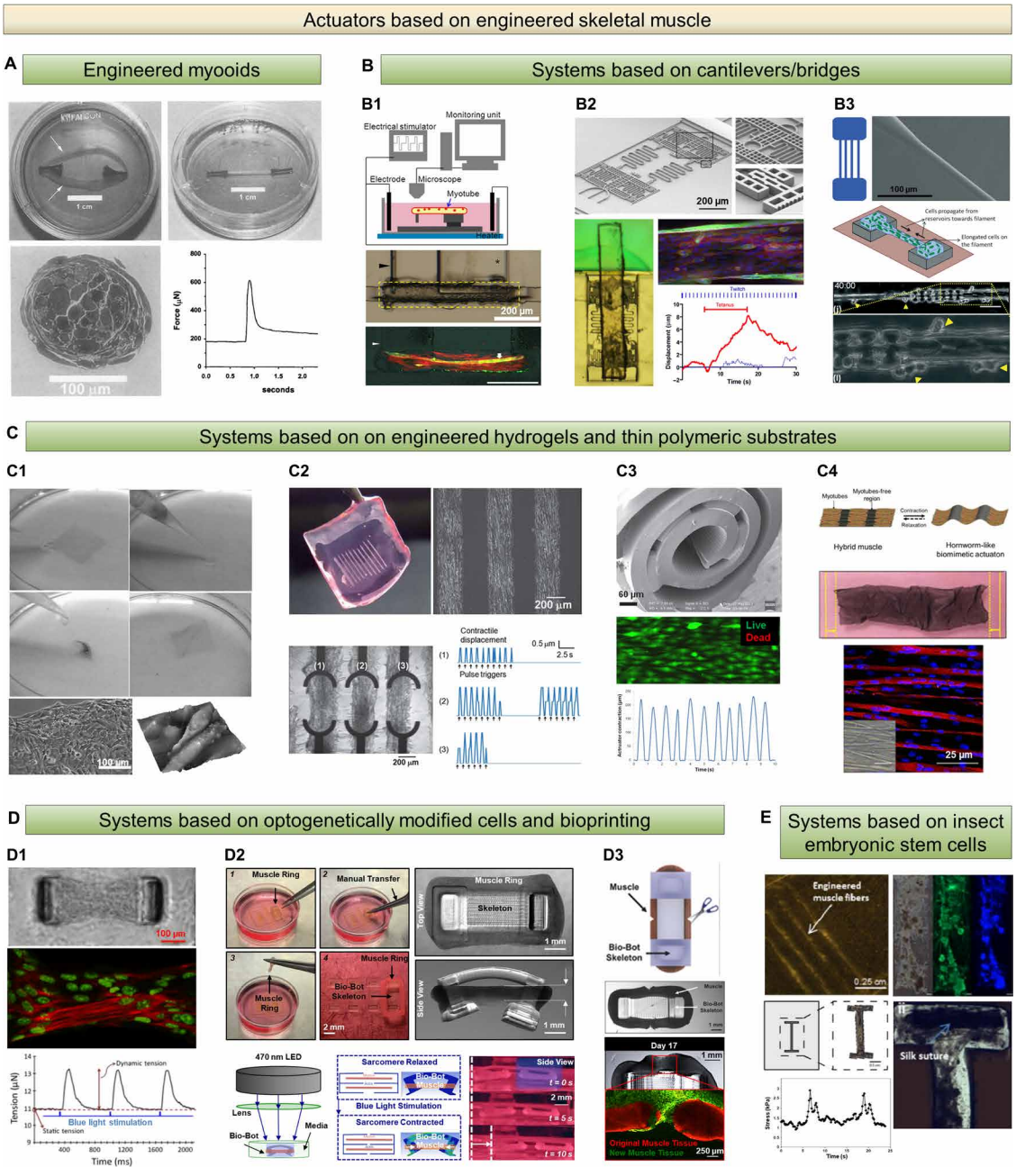

Fig. 4. Examples of general-purpose, scalable actuators based on engineered skeletal muscle. Different categories are represented: In vitro grown myooids (**A**), cantilevers/bridges (**B**), hydrogels and thin films (**C**), optogenetically modified cells and bioprinted structures (**D**), and insect embryonic stem cells (**E**). (A) Myooid engineered in vitro (top), its cross-sectional area (bottom left), and the peak twitch force recorded (bottom right) (*50*). Reprinted from (*50*) with permission from Springer. (B) Systems based on cantilevers/bridges. (B1) Contractile myotube assembled on a microfabricated cantilever (*53*). Reprinted from (*53*) with permission from Springer. (B2) Si-MEMS device seeded with skeletal muscle cells are able to contract after their differentiation in myotubes (*54*). Reprinted from (*54*) with permission from Springer. (B3) One-dimensional PDMS structure populated by myoblasts (*55*). Reprinted from (*55*) with permission from the Royal Society of Chemistry. (C) Systems based on engineered hydrogels and thin polymetric substrates. (C1) Ultrathin polylactic acid films cultured with skeletal muscle cells (*57*). Reprinted from (*57*) with permission from Springer. (C2) Fibrin gel provided with myotube line patterns and contracted through microelectrode arrays (*58*). Reprinted from (*58*) with permission from the Royal Society of Chemistry. (C3) Linear bioactuator based on a 3D rolled PDMS structure cultured with myoblasts (*63*). (C4) PEDOT/MWCNT sheet cultured with skeletal muscle cells and subjected to crawling-based locomotion (*64*). (D) Systems based on optogenetically modified cells and bioprinting. (D1) Optogenetically modified skeletal muscle microtissue tethered to elastic force sensors, recording its static and dynamic tension generated after optical stimulation (*66*). (D2) Top: Modular "ring" design enables ready transfer of skeletal muscle bioactuators to a range of flexible 3D-printed skeletons. Bottom: Light stimulation (left) of an optogenetic muscle-powered biobot drives muscle contraction (middle) and directional locomotion across a substrate (right) (*68*). (D3) Damaged muscle can self-heal completely within 2 days, restoring bioactuator force production (*69*). Reprinted from (*69*) with permission from Wiley. (E) Contractile tissue construct based on larval *M. sexta* muscle fibers provided with silk sutures and measurement of stress production (*73*). All images are reproduced or adapted with permission.

incorporated physical microgrooves in the gel substrate (ridge sizes of 50 and 100 μm), demonstrating an efficient alignment of muscle cells parallel to the microgrooves (*59*). In 2013, Ricotti *et al.* (*60*) proposed thin polyacrylamide gels and a synergistic combination of physical and chemical stimuli for 2D skeletal muscle engineering. The platform had the following features: (i) muscle-like stiffness; (ii) microtopography based on aligned microgrooved (width, 10 μm); (iii) biochemical factors resulting from the coculture of myoblasts and fibroblasts; and (iv) intracellular stimulation obtained by means of piezoelectric nanoparticles and ultrasound waves. The authors demonstrated that the combination of these physiochemical stimuli resulted in efficient muscle maturation and electrical activity. In the same year, Sun *et al.* (*61*) patterned aligned C2C12 myoblasts on PDMS thin films and differentiated them into myotubes to generate contractile actuators that generated peak twitch stress of ~10 kPa. Follow-up work by Duffy *et al.* (*62*) showed that both (i) the muscle cell species and (ii) the type of extracellular matrix protein used to pattern the muscle cells can affect differentiation efficiency and hence contractile force.

Vannozzi and colleagues (*63*) used PDMS thin films seeded with skeletal muscle cells and rolled in a 3D tubular structure by exploiting a self-assembly process (Fig. 4C3). They performed contraction tests of the rolled biohybrid actuator and used computer simulation to predict contraction forces by varying fabrication parameters. Results revealed that the resulting linear actuator may contract up to 25% of its length when seeded with cardiomyocytes and 0.12% when seeded with skeletal muscle cells. More recently, Webster *et al.* (*64*) fabricated and tested an electrocompacted and aligned collagen scaffold for engineering skeletal muscle. They recorded a velocity of 77 μm/s for the walking robot based on such scaffold. Kim *et al.* (*65*) developed a crawling robot based on multiwalled CNT (MWCNT) sheets coated with poly(3,4-ethylenedioxythiophene) (PEDOT) (Fig. 4C4). The authors demonstrated that the conductive nature of the biohybrid device permitted actuation at a faster rate by reducing the muscle refractory period (*65*).

Recent advances in tissue engineering have enabled the design and fabrication of 3D skeletal muscle bioactuators with superior force production and modularity than previously demonstrated. Sakar and colleagues (*66*) drove early advances by engineering 3D skeletal muscle microtissues tethered between stiff "tendon-like" micropillars (Fig. 4D1). Upon external stimulation, the bioactuator generated a passive tension force of 10.8 μN and an active contraction force of 1.41 μN. The cross-sectional stress (i.e., force normalized over cross-sectional area) produced by such biohybrid actuator was 0.112 kPa.

Three-dimensional skeletal muscle constructs have since increased in size to the millimeter scale, producing much greater passive and active tension forces, with demonstrated applicability as actuators for biohybrid robots (biobots). In 2014, Cvetkovic *et al.* (*67*) 3D-printed a flexible hydrogel "skeleton," composed of two stiff pillars (to mimic tendons) connected by a compliant beam (to mimic articulating joints). An injection molding process was used to engineer skeletal muscle, formed from the C2C12 myoblast cell line, around the skeleton's pillars. Electrically stimulated contraction of the muscle drove deflection of the soft robot, generating up to 1 mN passive tension force (0.84 kPa), 200 μN active tension force (0.17 kPa), and locomotive speeds of ~156 μm/s (over 1.5 body lengths per minute) (*67*).

Although this study demonstrated substantial advances in the functional performance of engineered skeletal muscle, directional locomotion could only be accomplished in biobots with asymmetric skeletons. Raman *et al.* (*68*) demonstrated that using optogenetically modified C2C12 myoblasts to fabricate bioactuators provides higher-level control over biobot locomotion, because the spatiotemporal resolution of light stimuli exceeds that of electrical stimuli (Fig. 4D2). In this study, a light-emitting diode was used to control 2D directional locomotion (310 μm/s, 1.3 body lengths per minute) and rotational steering (2°/s, 120°/min) in completely symmetric biobots. The noninvasive nature of light stimuli was further exploited as a mechanism of "training" muscle bioactuators during differentiation. A training protocol combining both static mechanical stimuli and dynamic optical stimuli was used to train skeletal muscle bioactuators to generate up to 2 mN passive tension force (3.2 kPa) and 300 μN active tension force (0.56 kPa). Raman *et al.* (*69*) further optimized this optical exercise protocol, and exploited its functional output, to drive remodeling and healing in damaged bioactuators (Fig. 4D3). Skeletal muscle bioactuators were lacerated to generate significant reductions in force, and an in vivo inspired healing strategy was designed to regenerate and remodel the damaged muscle. The optimal healing strategy—combining the recruitment of new myoblasts, concentrated controlled release of growth factors, and optical exercise—was shown to drive the complete recovery from damage of muscle force production within 2 days. This demonstration of the robust and resilient nature of bioactuators showcases the advantages of using adaptive biological materials as building blocks for biohybrid machines (concerning not only optogenetically controlled systems but also biohybrid devices in general).

The modularity and hierarchical structure of skeletal muscle in vivo is one of the primary features that make it useful for applications in biohybrid machines. To enhance the modularity and functional applicability of engineered muscle bioactuators, Raman and colleagues (*70*) have redesigned their bioactuators as muscle "rings" or "rubber bands" that can be stretched around flexible skeletons of any design, expanding the use of this bioactuator technology beyond the realm of locomotion into other functional behaviors, such as pumping. Muscle rings can be stacked on top of each other to achieve larger forces or even combined with 3D tissue rings composed of other cell types, such as neurons or endothelial cells, to generate more complex multicellular biohybrid machines (*71*).

The favorable properties of insect cells and tissues, mentioned earlier, have also been exploited to develop actuators based on insect striated (skeletal) muscle. In addition to their tolerance of changing environmental conditions, insect striated muscles are usually composed of a small number of single-celled fibers, and this simple organization may be recapitulated in vitro. It has been shown that networks of mature contractile skeletal myotubes can be grown from insect embryonic stem cells isolated from eggs of the tobacco hornworm, *Manduca sexta*, and that these myotubes differentiate into skeletal muscles (*72*). Metabolic analysis suggests that these growing cells do not rely on glucose as a primary energy source but instead are probably fueled by lipids and proteins.

A working biohybrid actuator based on these muscle fibers was developed by growing the cells on patterned biomaterial substrates or in narrow PDMS channels to promote alignment (Fig. 4E) (*73*). The cells were also attached to artificial tendons made from a variety of fibrous materials, including silk proteins. The insect muscle cells self-assembled into 3D structures, including loops and rings, without the need to be embedded in a hydrogel or another supporting matrix. The engineered muscles were typically 1 mm wide and 10 mm long, and the loops were 4 mm in diameter. When electrically stimulated, the biohybrid actuator contractions produced a stress of ~2 kPa and the system thrived without changes in media (composed of glucose,

proteins, and triglycerides) over at least 90 days, differently from mammalian cell systems, which require medium exchanges every 2 to 3 days to keep them viable. The blocked stress generated by these muscle constructs is still low compared with native muscle, but it could be notably improved by increasing the cell density and alignment by mechanical and electrical stimulation. The bioactuator was able to maintain sustained contractions in different conditions of pH (5.5, 6.5, and 7.5) and temperature (15°, 26°, and 37°C), thus demonstrating its robustness. In addition, the muscle constructs had a self-healing capability that could be exploited for anchoring bioactuators within devices. A 1-mm defect introduced into a preformed muscle partially repaired itself within 1 month, and contractions resumed in the repaired region. This repair involved the migration of muscle cells into the defect toward the opposite side of the resected region accompanied by cell elongation. Movie S5 shows the concepts and performance of biohybrid systems based on engineered skeletal muscle tissues.

BIOHYBRID ACTUATOR PERFORMANCE AND COMPARISON WITH FULLY ARTIFICIAL TECHNOLOGIES

In robotics, the requirements for actuator performance can range widely, depending on the target application, size, power, conditions, and time of operation. Despite such variety, certain performance standards (or figures of merit) have been identified and are commonly used to compare different actuator technologies. They are based on quantitative factors, such as output force (N) or stress (kPa), stroke (m), power/weight ratio (W/kg), work capacity (N·m), energy density (J/cm^3), operation frequency (Hz), and efficiency (%). In addition to these numbers, other quantitative or qualitative factors must be considered when choosing a specific actuation strategy for a certain task, for example, weight, cost, motion control energy storage, flexibility, and robustness (*4*).

One of the most powerful features of biohybrid actuators is undoubtedly their inherent scalability at small dimensions. It is widely recognized that traditional actuators (e.g., electromagnetic motors) cannot be much reduced in size without losing the ability to produce detectable forces and torques. This is due both to difficulty in manufacturing and assembling single motor components at small dimensions and to physical scaling laws that imply an exponential decrease of the output force with size. Only piezoelectric motors can be scaled rather efficiently in the minidomain (from a few millimeters to a few centimeters) and they just stay at the boundary of the microdomain (below 1 mm) (*74*), but they cannot approach the level of miniaturization of living motile or contractile cells. Figure 5A shows a force versus size graph for both biological microactuators (motile microorganisms, cardiomyocytes, and single contractile skeletal myotubes) and some artificial actuation technologies.

Some highly miniaturized piezoelectric motors are nowadays available, such as the SQUIGGLE (New Scale Technologies; size, 1.8 mm by 1.8 mm by 6 mm) or the PiezoWave (PiezoMotor AB; size, 14 mm by 7.2 mm by 4.4 mm). Few attempts to overcome the 10^{-2} cm^3 "barrier" have been pursued (*7*) but without much success: One of the smallest existing ultrasonic motors reported to date is a combined axial-torsional standing-wave motor with a diameter of 0.25 mm and a length of 1 mm, fabricated by laser micromachining. However, its real total main dimension, including the preload mechanism, is 2 to 3 mm, and it can produce only a small torque (~13 nN·m) (*75*).

Mammalian contractile cells are several orders of magnitude smaller than the smallest piezoelectric motors: Cardiomyocytes (~100 μm by 100 μm by 10 μm), during their beating, generate a contraction force of ~10 μN (*76*), whereas single skeletal myotubes cultured in vitro (~300 μm by 50 μm by 10 μm) are currently able to develop a contraction force of ~1 μN (*53*). Bacteria and other motile microorganisms are very small (1 to 3 μm in diameter); their flagellar motor is composed of torque-generating units whose power production capability even exceeds the one of actomyosin motor (*77*). The ability to efficiently produce detectable forces at small scales is the most exciting feature of biohybrid actuators, which make them attractive for all the applications in which microrobots/nanorobots endowed with onboard propulsion systems are desirable (e.g., in the field of targeted therapy). So far, bacteria dominate the field of biohybrid robots, which could be delivered to the bloodstream or other fluidic regions inside the body for performing therapy (*16*–*26*). However, cardiomyocytes and skeletal muscle cells also start to be seriously considered for this purpose (*10*, *12*, *43*).

Figure 5B shows a classification of both artificial actuators and general-purpose biohybrid ones in terms of stress and stroke. The figures of merit for biohybrid actuators have been derived from data available on stress and video-extracted data, concerning stroke. Few recent references for the different categories are reported. Current biohybrid solutions are not really competitive with artificial actuators and are rather far from natural muscle, in terms of overall performance. This is particularly true for skeletal muscle–based systems, which are still hampered by both a low stress production (up to ~2 kPa, in comparison with the hundreds of kilopascals of natural muscle) and a low stroke (~1%).

Cardiomyocyte-based systems show the best performance, with stroke values almost matching the ones typical of natural muscle and pneumatic actuators and much higher than the ones typical of piezoelectric, magnetostrictive (MS), and SMA actuators, conducting polymers, and polymer gels. For cardiomyocyte-based systems, the stress produced does not match the one of natural muscles but is roughly only one order of magnitude smaller and is comparable with the one produced by piezoelectric multimorphs and piezoelectric inchworm motors. The good performance of these systems is highly dependent on the cell source (primary cells, opposed to the cell lines used for many skeletal muscle–based systems). This provides an inherent advantage but also clear drawbacks, such as the need for sacrificing animals to obtain cells, with consequent ethical, cost-related, and usability-related issues. Moreover, as outlined in the previous sections, actuators based on cardiac cells are featured by a scarce controllability; this is a crucial issue that may be faced by evolving optogenetic techniques (*45*).

The potential of general-purpose scalable biohybrid actuation technologies in robotics must be more deeply analyzed and discussed. Although such potential is clear at the microscale due to a lack of artificial alternatives, the same considerations do not apply at the macroscale, where several other solutions, already engineered, are available. Assuming that future technological efforts will permit to develop biohybrid actuators matching or even slighting outperforming natural muscle, some fully artificial technologies are likely to keep a substantial advantage over biohybrid ones (Fig. 5, B and C). Natural muscle is outperformed by pneumatic/hydraulic systems in terms of stress, by dielectric elastomers in terms of stroke, and by several other technologies (including piezoelectric motors) in terms of power density and efficiency (*4*, *74*, *78*). However, other aspects must be taken into account.

In general, actuators must be considered as part of a system that also includes a power source. In addition, in some cases, a device converting the power source into a form that the actuator can accept and

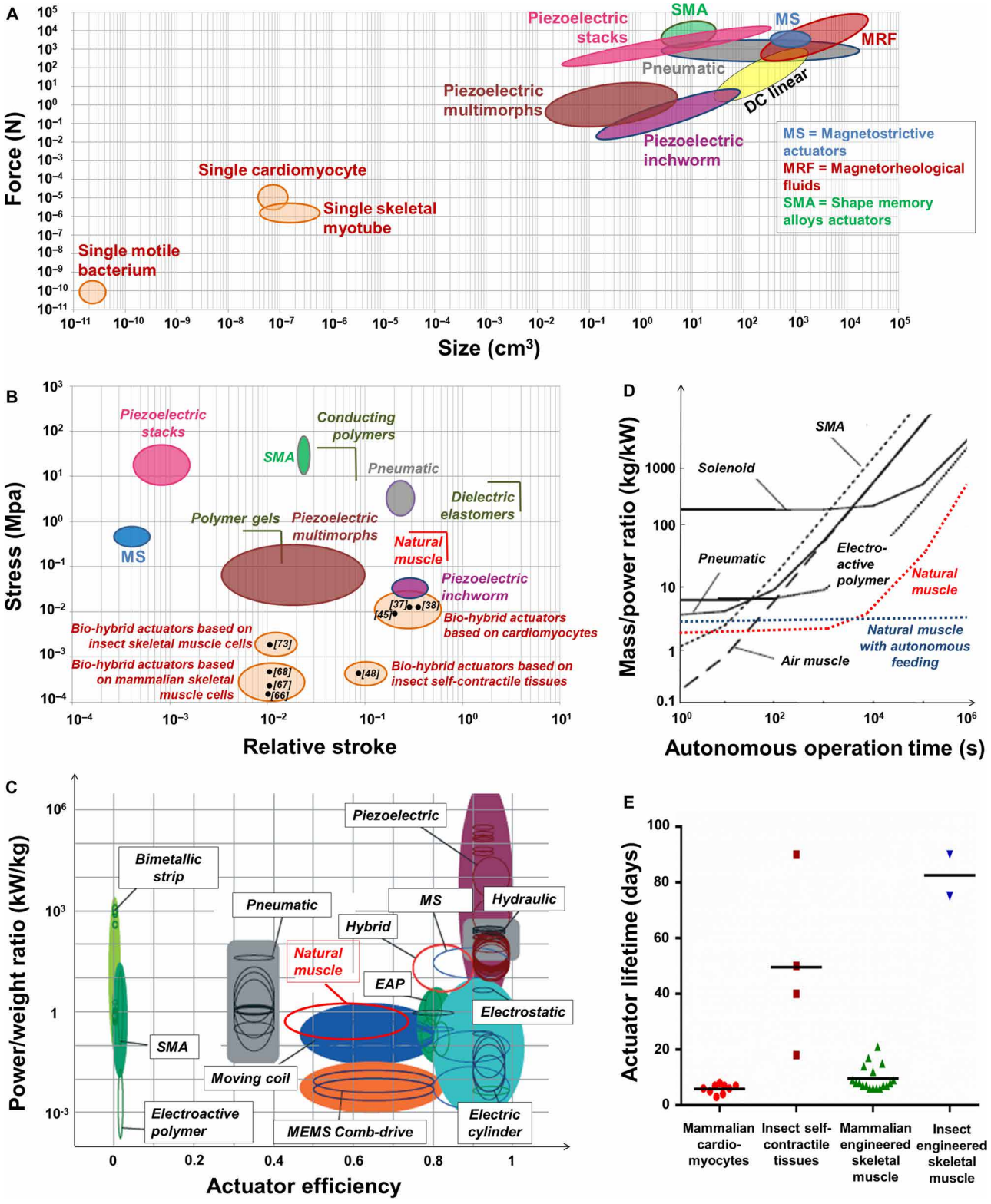

Fig. 5. Performance of biohybrid actuators and comparison with fully artificial counterparts. (**A**) Actuator force output versus its overall size. (**B**) Plotting of both artificial and biohybrid actuators in terms of stress produced versus relative stroke. Few references are reported in brackets for biohybrid actuators. (**C**) Plotting of artificial actuators and natural muscle in terms of power/weight ratio versus actuator efficiency (*4*). (**D**) Mass/power ratio, which takes into account both actuator and power source mass, versus actuator autonomous operation time. Some artificial actuation technologies are compared with natural muscle, assuming no periodical feeding (red line) and periodical autonomous feeding (blue line). Data in (C) and (D) reprinted from (*4*) with permission from Wiley. (**E**) Lifetimes of "general-purpose" biohybrid actuators reported in the literature for the different categories analyzed in this paper.

another device converting the motion produced by the actuator into another one, more appropriate for the target application, must be also considered. Thus, a fully autonomous system must be provided with appropriate converters and an embedded energy source, featured with a proper mass, allowing the actuator operation for the desired time frame. This scales up the overall system dimensions, when long-term autonomous tasks are needed.

Figure 5D shows a plot of the mass/power of different actuating systems as a function of the overall operation time during which they are assumed fully autonomous. SMA and pneumatic systems need a relatively small actuator/converter mass but require a high amount of energy that rapidly increases their mass/power ratio over time. Solenoids, instead, have a large initial mass, but their energy demand is lower. In this framework, natural muscle (and consequently the biohybrid actuation paradigm) has high potential. In an adult human, about half of the body consists of muscle tissue. We can thus roughly consider that its operation is supported by the other half of the body. This has a clear advantage over other systems (such as pneumatic or hydraulic), for which the energy source often weighs many times the total actuator mass. The mass/power ratio over time for natural muscle can be derived assuming that it is fed with a high-glucose solution (the solubility of glucose in water is ~190 mg/ml) and has an aerobic transduction efficiency of ~1000 J per gram of glucose (red line in Fig. 5D) (*79*). For relatively long-lasting tasks, muscle is more advantageous with respect to all artificial technologies. Furthermore, it can be envisaged that muscle-equipped robots could be provided with a relatively small self-contained power supply (a small volume of high-glucose solution) and with an autonomous refueling mechanism (e.g., based on a chemical energy conversion system, reflecting our digestive/metabolic apparatus). Such an "autonomous feeding system" would imply an initial additional mass but would allow the mass/power ratio to keep constant for an indefinite amount of time (blue line in Fig. 5D), opening exciting scenarios in mobile and space robotics.

In this framework, the lifetime of biohybrid actuators obviously plays a very important role. Figure 5E shows that, at present, only insect-based systems can be kept alive and operative for several months (up to 90 days) (*46*, *73*). Actuators based on mammalian cells, instead, typically remain viable and functional only for a few days or weeks. To harness the advantages of biohybrid technologies over artificial ones in the macrodomains of robotics, bioactuators must advance not only in terms of overall stress and stroke but also in terms of medium- and long-term stability of the muscle construct.

Besides their high miniaturization ability and their potentially low mass/generated power ratio over time, there are other factors that make biohybrid actuation solutions really promising for robotic applications (*80*). One of them is the unique ability of living tissues to self-heal: When subjected to damage, a bioactuator has the potential to self-regenerate in a few hours/days, recovering its original performance. This property has been scarcely exploited in biohybrid actuation (*69*, *73*), but it may pave the way to actuators robustly resilient to damages. Muscle tissues can dynamically respond to changing environmental loads, even adapting their size and performance. On the one hand, this is an issue (changes in the system imply a less predictable and controllable behavior), but on the other hand, it is also an opportunity, because it adds enormous flexibility to the actuation unit. In addition, muscle tissues operate silently; use a range of fuel sources that are inexpensive and readily available (mostly sugars and fatty acids); generate biodegradable (eco-friendly) substances during fuel-work conversion; and, in the case of mature structures, have sensors embedded (the muscle spindles) that may be used as stretching sensors or even to endow the biohybrid actuation unit with more complex proprioception abilities. Last, muscle-based actuators have the potential to efficiently modulate their stiffness, by recruiting a different number of myofibers or by synergistically acting to stiffen/soften a joint. This property is highly relevant in robotics, and, although fully artificial technologies are being developed and refined for this purpose (*5*), bioactuators may represent more elegant and compact solutions to develop compliant robots with inherent control over mechanical impedance.

OPPORTUNITIES, OPEN CHALLENGES, AND MISSING BLOCKS

Microorganism-based biohybrid actuators: Promises for medical applications

Biohybrid actuators based on bacteria and other motile cells have the potential to bring the classic movie *Fantastic Voyage*, in which a miniaturized submarine and its crew are injected into a patient's bloodstream to destroy a blood clot, closer to reality (*17*). To enable biobots to precisely and locally reach a clinical target within the body, three fundamental specifications typically integrated in robotic agents must be considered, namely, (i) sensing, (ii) some level of computation, and (iii) actuation. Thus, actuation is only a part of the story: It is of crucial importance, but it must be enriched by additional functionalities. The unique advantage of biohybrid systems over fully synthetic microswimmers that are emerging in literature (*81*, *82*) is the ability of living cells to detect and respond to various types of environmental stimuli without additional components.

Different control methods have been devised for steering biohybrid microswimmers toward the target area (*10*). Electric steering, obtained through galvanotaxis or electrophoresis, is limited to lab-on-a-chip and other in vitro scenarios. Optical control methods (used to steer phototactic bacteria and algae) are attractive, but body tissues are generally poorly transparent to light; thus, in vivo applications are not straightforward, especially in deep tissues. Ultrasound has not been extensively used in microrobot control due to poor reliability and reversibility. Magnetic control seems at present the most promising approach because magnetic fields can efficiently and safely penetrate body tissues. Magnetotactic bacteria are an attractive option, because they are naturally endowed with magnetic nanocrystals; however, other living cells can be artificially magnetized, for example, by embedding them with iron oxide nanoparticles or attaching them to magnetic substrates. Some open challenges concern the hardware used to generate magnetic fields: Electromagnets can generate magnetic fields rapidly and precisely by controlling the input current; however, currents can heat the system and the whole workspace, altering cell behavior or even affecting cell viability. Permanent magnets avoid heating, but they need to be physically moved to modulate the field. Cooling systems for electromagnets, superconducting coils, or robotic platforms for reliably moving permanent magnets are possible routes to be investigated. In some scenarios, selective and parallel control of multiple biohybrid microrobots could be desirable. This may be achieved through heterogeneous biohybrid robot designs (*81*) and selective magnetic disabling (*83*).

Chemical control methods, governed by chemical diffusion, generally have poor temporal resolution. In addition, they are characterized by a relatively large stochasticity. This strategy is promising when chemoattractants are naturally available in the target area: Biochemical signals released, for example, from tumor cells, can elicit a response from the biohybrid device when it is in proximity of the target, thus refining its motion. This approach and analogous "passive" ones

(e.g., thermotaxis and aerotaxis) look attractive only in combination with other "active" strategies. For example, magnetic fields have been used to steer bacteria, whereas aerotaxis has been subsequently exploited to guarantee their deep colonization of the tumor mass (*26*). Currently, the combination of long-range active "robotic" control strategies and short-range passive "biological" reactivity represents the most promising paradigm. Further developments should exploit all of the variety of living elements. For example, a certain level of biological autonomy (e.g., physiological obstacle avoidance ability) would be desirable to compensate for the lack of spatial resolution and to gather the required information to implement closed-loop control. Sense-compute-actuate molecular units may be engineered by using DNA nanotechnology approaches. This would allow microorganisms to be redesigned with additional artificial/synthetic components for a given mission.

Another missing block in this framework (and in microrobotics in general) is the lack of safe and reliable imaging to track the microswimmers in vivo. This would allow image-based feedback control in some applications. In general, real-time tracking, feedback, and physiological registration of such microswimmers inside the in vivo workspace are very challenging. Because there are many uncertainties and dynamic changes in such workspace, feedback control would be desirable for achieving safe and optimal microswimmer operation (*84*). Reliable imaging would also allow monitoring of the efficacy/safety of a therapy over time. Novel contrast agents or different nanotechnology-based strategies should be devised for this purpose. Last, a disadvantage of using microorganisms is the pathogenicity or scarce biocompatibility of some strains. As mentioned earlier, nonpathogenic forms can be found/engineered (several bacteria types, for example, the ones constituting our microbiota, live in our body), or alternative motile cell types can be used.

Biohybrid actuators based on explanted muscle tissues: Narrow margins of improvements?

The issues affecting biohybrid actuators based on explanted whole-muscle tissues described earlier, especially the poor stability out of the body, make these systems rather unsuitable for applications in robotics at present. One promising route is to use the natural extracellular matrix of explanted muscle as a scaffold for subsequent recellularization (*85*). This would provide a natural vascular bed to build 3D systems with a mixed top-down and bottom-up approach. The decellularization process can damage important matrix features and should be optimized. Besides the obvious ethical issues involved, with this approach, the possible actuator architectures would remain limited to those available in nature.

General-purpose and scalable biohybrid actuator routes toward optimization

The general-purpose and scalable biohybrid systems described earlier are grounded on a bottom-up approach and, as highlighted already, require several research efforts directed to their optimization to make them competitive with other fully artificial actuation technologies (Fig. 5). The different routes to be explored to target such optimization are discussed in the following subsections.

Biological advancements: Cocultures and stem cells

Improving the scalability and performance of biohybrid robots remains a major challenge: Addressing it will surely require advancements in the engineered biological components. For example, because these actuators are composed of living cells, the types of cells used, generating these cells at large scale and low cost, and maintaining these cells over long periods of time are areas that need further development. Cardiomyocytes are terminally differentiated cells: To avoid ethically questionable and inconvenient harvesting from animals, they may be differentiated from pluripotent stem cells. Although there have been improvements in the differentiation protocols to decrease the cost and increase the yield of cardiomyocytes (*86*), the process remains expensive and challenging to scale up to the billions of cells needed for large actuators. Skeletal muscle stem cells and myoblasts can be expanded more easily and at lower cost, with cell lines such as murine C2C12 myoblasts having been expanded for decades using relative inexpensive media, for example, based on Dulbecco's modified Eagle's medium with fetal bovine serum supplementation. However, it is difficult to have all the myoblasts fuse and differentiate into contractile myotubes, a factor that limits the density of muscle that can be generated. Natural muscle is characterized by a very efficient packing of biomolecular motors: ~95% of a healthy muscle cross-sectional area consists of tightly packed actin and myosin proteins, organized in a hexagonal lattice. Currently cultured muscle tissues suffer from a significantly lower volumetric efficiency: Contractile proteins typically represent 5 to 10% of the value found in an adult healthy muscle (*80*).

For both cardiac and skeletal muscle, other cells play important roles: Fibroblasts, neurons, vascular (endothelial) cells, and other support cells are crucial for organizing and maintaining the muscle tissue architecture. Deriving and expanding these additional cell types, as well as the specific numbers needed, is an area of continued research to improve both the contractile performance and the lifetime of muscle constructs and consequently of biohybrid actuators. Really few examples of bioactuators based on cell cocultures have been described so far (*32*, *60*, *71*), and no papers have described systems composed of several cell types, trying to reproduce all the main muscle interfaces (vascular, neural, and myotendinous ones). However, multicellular (heterotypic) systems have been recently indicated as possible transformative elements (*9*, *13*). Pluripotent stem cells are promising sources to achieve complex and multiphenotype systems.

Technologies to boost muscle development

The contractile force of in vitro cultured muscles is directly related to the expression of actin and myosin, the main elements of the cell contractile machinery. Accelerating or enhancing the expression of such proteins can be an effective way to increase biohybrid actuator performances. In addition to myosin and actin expression, myogenesis is regulated by a complex signaling pathway with multiple entry points and steps. Thus, controlling the many extra- and intracellular molecules and proteins involved in this process would also be powerful. This can be pursued through different routes and by drawing from materials science, mechatronics, biotechnology, and nanotechnology.

Materials can also be engineered from several viewpoints to maximize muscle maturation. For example, it is known that an anisotropic surface topography effectively promotes myoblast alignment along one direction, which is a prerequisite for the formation of multinucleated, uniformly oriented contractile myotubes able to produce a net force in that direction. The literature on this topic is very rich and sometimes discordant. However, as a rule of thumb, materials provided with ~10-μm-wide grooves/ridges, spaced by ~10 μm, and with a depth of 1 to 5 μm are effective in aligning myoblasts and in promoting their differentiation in myotubes (*9*, *60*). However, submicrometric patterns seem to be more effective than micrometric ones in promoting the expression of contractile proteins (*87*). The knowledge needs to be partly clarified, especially for poorly investigated cell types (e.g., insect ones), to identify the most effective parameters to be used. Substrate stiffness

also plays a key role in muscle development: The stiffness range for the differentiation of C2C12 murine myoblasts is 10 to 15 kPa (*88*). In case other cell types would be used for building the desired biohybrid actuators, similar reference values should be identified.

Besides scaffold optimization, cell-instructive inputs can be provided by means of external mechatronic systems. It is known that uniaxial cyclic mechanical stimulation and pulsed electrical stimulation can enhance/accelerate the expression of proteins and transcription factors involved in myogenesis. Low-intensity pulsed ultrasound also demonstrated its potential in boosting muscle development when applied at specific frequencies and intensities (*89*). The optimization of these stimulation protocols and the investigation of synergies among multiphysical stimulations, biomaterial features, and biochemical inputs are hot topics in regenerative medicine and may also contribute to the optimization of biohybrid actuators.

Further strategies to boost muscle development rely on cell engineering techniques. They can be based on biotechnological modifications (e.g., transfections with bacteria, virus, or plasmids), in which target muscle cells are provided with foreign genes that modify/ameliorate their behavior, or nanotechnology-based strategies, in which functional nanoparticles are used as intracellular transducers to stimulate cells, thus further accelerating/enhancing the expression of key genes and proteins (*60*).

Upscaling: Toward 3D systems

For biological actuators, generating more force requires engineering larger muscle tissue. This is mandatory if the target is to use biohybrid actuators in the macrodomains of robotics. Although having more cells is one part of this problem, equally important is a vasculature system to provide mass transport of nutrients and waste in 3D to support cell metabolism. Without a proper perfusion system, metabolic exchanges are guaranteed only by diffusion, which constraints engineered muscle diameters to ~100 to 150 μm (*50*).

The formation of vasculature is a challenge that has faced tissue engineering since its inception and has yet to be solved. However, there are a number of new technologies being developed to engineer vasculature. Recently, 3D bioprinting has emerged as a viable fabrication platform to engineer large tissue constructs with integrated vasculature (*90*). For example, sacrificial polymers can be 3D-printed into complex networks, and then cell-laden hydrogels can be cast around this and the sacrificial polymers can be removed to create fluidic networks to perfuse the tissue construct (*91*). It is also possible to directly 3D bioprint cell-laden hydrogels into complex tissue architectures, including constructs based on the embryonic heart and skeletal muscle bundles (*92*, *93*). Three-dimensional bioprinting, together with other advanced biofabrication approaches, is thus emerging as a promising solution to engineer large, vascularized muscle tissue constructs that will be required to power and actuate large soft robotic devices.

An alternative route to scaling up biohybrid structures may include the development and assembly of several parallel thin or ultrathin deformable films (*12*) seeded with the desired cell types. These films are used as units of a multisheet 3D structure. This would keep the relative ease of a 2D culture, moving the complexity to a 3D sheet assembly task, which would require advanced micromanipulation strategies or smart multisheet fabrication and seeding procedures.

Optimization of triggering strategies

Contraction of most general-purpose biohybrid actuators described in this paper is controlled using electrical pulses to depolarize myotubes. Although usually reliable, electrical stimulation involves some drawbacks, including electrolysis, bubble formation, and electrochemical cell damage. In addition, it requires placing electrodes close to cells and an external power source such as a battery to generate triggering signals. Although this is acceptable at large scales, the same does not apply if small (submillimeter) autonomous bioactuators are needed, in which case biohybrid robots must be kept in confined and controlled environments, such as the ones typical of lab-on-a-chip applications.

Optogenetics recently emerged as a very powerful means to overcome the drawbacks of electrical triggering: Optical stimulation is safer at a cellular level, avoiding any electrolysis and electrochemical damage–related effect and keeping the triggering source (a laser) external to the biohybrid unit. This allows miniaturization of the system, which does not need to integrate any battery: The light can be applied by a user through remote optical fibers or light-emitting diodes (LEDs). Optogenetic modifications should be further evolved to not hamper the contractility of skeletal muscles and possibly to make cardiac cells fully controllable, by inhibiting self-beating activity and boosting optically controlled one. This paradigm may also be developed to provide biohybrid systems with several degrees of freedom of controllability, for example, by inducing the expression of different light-responsive proteins in muscle cells, able to be triggered by different light wavelengths.

Another strategy for triggering the contraction of in vitro developed muscle constructs by using light has been recently reported (*94*). In this paper, near-infrared radiation was exploited in combination with gold nanoshells to promote photothermal conversion and consequent mild intracellular hyperthermia, which remotely triggered the contraction of striated muscle cells. Although intriguing, this phenomenon lags behind other triggering methods, because the intracellular temperature increase needed to incept contraction (~5°C) can be reached only with prolonged laser stimulation (~40 s). The temporal resolution of this approach is thus currently insufficient for an effective control of biohybrid units.

In general, phototactic guidance of biohybrid robots looks promising, but it cannot be considered an optimal general-purpose triggering solution. With respect to electrical control, a light-based one does not eliminate the driving apparatus but only moves it from the cell proximity to the external (remote) space. This is advantageous but can raise controllability issues: A phototactic steering implies the need to move the laser (or another cell source) together with the robot or to develop a complex array of LEDs to be activated in a properly temporized fashion. This may be unfeasible for certain applications. In addition, living tissues are not transparent to light, especially in certain wavelength ranges; thus, this technology can be used in vivo only to drive robots placed at a few millimeters from the skin surface.

Tendon-like interfaces for force transmission

In many natural biological systems, movement is generated at articulating joints in the skeletal system. This movement is driven by the contraction of skeletal muscle, which is tethered to bones via tendons. The skeletal muscle–based robots described above rely on biochemical mechanisms for tethering tissue to an underlying substrate or on purely mechanical tethering—the muscle is stretched around synthetic skeletons and kept in tension. This poses a notable contrast to the tendon-based triggering observed in natural biological systems and could diminish force transmission from the bioactuator to the underlying substrate or skeleton.

There have been limited efforts to engineer tendon-like interfaces for skeletal muscle, and most approaches have focused primarily on applications in regenerative medicine. Early advances by Larkin *et al.*

(*95*) demonstrated that cocultured constructs of tissue-engineered muscle and tendon could produce robust myotendinous interfaces that withstood strains beyond the physiological range. In addition to multicell approaches, efforts to manufacture tissue scaffolds to promote the formation of interfaces have also met with good success. For example, Ladd *et al.* (*96*) successfully achieved layered scaffolds that allow engineering gradual interfaces between different types of tissues.

Engineering myotendinous interfaces for applications in soft robotics will likely require further investigation into these forms of functionally graded multicellular systems, with emphasis on forming interfaces with the synthetic materials commonly used for these applications. A promising recent advance reported by Merceron *et al.* (*97*) demonstrated the construction of a muscle-tendon unit using 3D-printed functionally graded scaffolds seeded with C2C12 skeletal muscle cells and NIH/3T3 fibroblasts. Such an approach could potentially be integrated within 3D-printed skeletal muscle–powered robots (*67*–*71*), enabling more efficient force transfer from the bioactuator to the flexible skeleton. Successful integration of well-matched muscle-skeleton attachments (apodemes) in future biohybrid robotic devices could greatly enhance functional outcomes and drive forward real-world application of these machines.

Life-sustaining systems or insect cells?

If mammalian cells are used to develop biohybrid units, an important issue to be considered is how to keep the biohybrid structure viable and functional once it is removed from the incubator. Except the envisaged medical applications in which biohybrid robots are delivered in the body (in which they would be kept at 37°C and may find suitable nutrients to self-feed), in all the other contexts, they are subjected to varying temperature, CO_2, and pH levels, as well as to possible contaminations by microbial agents. A proper life-sustaining system is thus mandatory to maintain long-term functionality of mammalian cells in practical engineering- and robotics-related scenarios. Such systems should be designed to function as integrated, light, and flexible bioreactor: They should keep cells isolated from the external world, maintain mammalian cells at 37°C, and keep them immersed in a nutrient-supplied liquid medium, periodically renewed in a manual or automatic fashion, for example, through a fluidic system connected to the autonomous feeding system. Such a system would be composed of several mechatronic components; however, it should guarantee a high deformability to prevent hampering overall bioactuator contraction efficiency. No papers on this topic have been described so far. However, recent achievements on stretchable electronics (e.g., based on graphene) (*98*), multifunctional materials for robot skins (*99*), and miniaturization of bioreactors for tissue engineering (*100*) suggest that the key technologies have reached a good level of maturity to address this challenge.

As described above, insect tissues are highly promising for the development of robust biohybrid machines: Even if this field is less explored, a major and well recognizable advantage of this approach is that insect-based actuators do not need life-sustaining systems or they need very simple ones. Because insects are generally small and they have an open circulatory system, individual tissues are adapted to withstand extreme and rapidly changing environmental conditions. This has allowed insects to dominate niches that are not tolerated by other animal groups. Also, because there are so many insect species, it should be possible to engineer bioactuators with specific properties through careful selection of donor stem cells.

For example, silver ants in the Sahara are active at surface temperatures up to 63°C and tolerate body temperatures up to 54°C. At the opposite extreme, glacial midges (*Diamesa* sp.) walk at temperatures as low as −16°C (*101*) and the geometrid *Operophtera bruceata* flies at field temperatures as low as −3°C. Many insects can recover from temperatures well below freezing (*102*). Presumably, these species would be a very good source for myocytes tolerant of extreme temperatures.

Water and salt balance is particularly challenging for small terrestrial animals, and yet, most summer insects have hemolymph osmolalities ranging between 400 and 600 mosmol kg^{-1}, values that could be fatal to some mammals. Winter buildup of polyols and other solutes in insects can exceed 3000 mosmol kg^{-1}. Such species could provide cells that can survive in specialized solutions and even be stored frozen for extended periods.

A particularly acute problem for engineering mammalian tissues is the need for a finely branched vasculature; tissues with a diameter larger than about 100 μm from a blood vessel are subject to anoxia and become necrotic. Humans suffer acute effects when oxygen concentration is reduced by half (to ~10 kPa), and mammalian cells die when exposed directly to normal air. In contrast, insects do not have a vasculature, and tissues receive environmental oxygen and other gas exchanges through an open tracheal system. Most insects maintain posture and responsiveness at oxygen pressures as low as 2 to 3 kPa (*103*), and they can recover from up to days of anoxia (*104*). Insect bioactuators should be capable of operating in most ambient conditions and capable of surviving extreme oxygen deprivation. Insects are also famously resistant to general toxicants (*105*) and ionizing radiation that would almost immediately disable a human or other mammal (*106*).

Last, insects are capable of extraordinary work and power output. A handful of honeybees equal in mass to a 40-W bulb generates almost as much heat, and insect flight muscles produce the highest sustained power output per mass of any animal (*107*). The myocytes that form asynchronous insect flight muscles are therefore an excellent candidate for engineering long-lasting and powerful bioactuators. The molecular networks that control surface interaction signaling and tissue assembly in insects have been studied in detail (*108*, *109*), but further knowledge is needed to identify critical and accessible elements of these pathways and thus to achieve effective insect muscle engineering. The same applies to the development of muscle-tendon interfaces (insect apodemes) (*110*), with the perspective to grow complete myotendinous actuators that self-assemble onto integrated insect-based biohybrid robots.

CONCLUSION

Exploiting the unique features of biological motors for producing controlled motion in artificial machines is an exciting and evolving challenge. The research field of biohybrid actuators is rapidly progressing, mainly driven by ambitious scientific hypotheses involving molecular biology, biotechnology, materials science, and mechatronics, in a deeply interdisciplinary paradigm.

Biohybrid actuators can be classified as (i) application-oriented, nonscalable devices and (ii) general-purpose, scalable devices. The first category includes systems based on bacteria and other motile cells and devices based on explanted whole-muscle tissues. Bacteria-based devices look very promising for targeted therapy at small scales, because of dimensions compatible with navigation in the human capillaries, whereas devices integrating whole explanted tissues show low flexibility and poor lifetime at present, which make their future use in robotics not likely. The second category of bioactuators relies on a bottom-up approach and includes systems based on cardiomyocytes, insect self-contractile tissues, and engineered skeletal muscle tissues

(from both mammals and insects). Cardiac cells and self-contractile insect tissues have interesting properties (cardiomyocytes exert a large stress and insect tissues show high robustness and extended lifetime), but their inherent self-contractility hampers their use in robotics due to a scarce controllability. This issue may be solved in the future by evolving optogenetic control strategies, but other possible drawbacks related to the need for reliably moving the light source together with the robot and to tissue-mediated light adsorption must be taken into account. Mammalian and insect skeletal muscle–based devices are among the most promising systems in this field, but they currently lack both biological and technological optimization steps to become competitive with fully artificial technologies.

At present, the main motivations for pursuing the development of biohybrid actuators are the ability to build highly miniaturized robots, with a volume several orders of magnitude smaller than the smallest existing piezoelectric motors; the opportunity to keep the actuating system mass/generated power ratio low with respect to all artificial technologies, especially for long-term tasks; and the ability to exploit the unique features of living cells, such as self-healing capability, dynamic response to changing environmental conditions, silent operation, use of inexpensive and eco-friendly fuel, and presence of embedded sensors (muscle spindles).

As we move toward the full exploitation of the biohybrid paradigm potential, both basic science and practical engineering issues will emerge. A lack of a fundamental understanding of certain biological phenomena is missing, especially concerning the ultimate control of muscle size, shape, and interactions with other tissues. From an engineering viewpoint, missing blocks include biofabrication approaches that can build macroscale structures with microscale features, technologies to boost muscle development and to optimize its control, development of tendon-like interfaces, life-sustaining systems, and advanced mathematical models. Most research efforts dealing with cell and tissue engineering are exquisitely experimental and often based on a trial-and-error approach. This also applies to the field of biohybrid actuators, in which modeling has had a rather small role so far. However, as in several other engineering branches, this research field would strongly benefit from advanced mathematical models, optimization algorithms, and possibly software tools for optimizing the design and for predicting the performances of future biohybrid systems. Current state-of-the-art efforts mainly focused on the mechanical deformation of polymeric thin films (mostly considered as cantilevers) due to muscle cell contraction' (*111*–*113*). More complex multiscale models (e.g., based on evolutionary algorithms) may permit optimal configurations and combinations of contractile biohybrid units, using an integrative design approach. This may launch a new line of optimized biohybrid technologies and be key for filling the gap between biohybrid actuators and fully artificial ones.

Filling these gaps would allow this field to evolve from the "art of possible" to the science of "reliable manufacturing." Systematic design tools and quality control metrics are also required, as well as appropriate control experiments and quantitative performance standards. To this purpose, both education and research endeavors must be evolved to support the developing field of biohybrid robotics, which may solidify in a series of practical scenarios, in the future. Possible concrete short- and mid-term applications of biohybrid actuators are specialized platforms for investigating agonistic-antagonistic muscle contractions; proof of concepts of miniaturized therapeutic robots; and implantable, reconfigurable biohybrid medical devices. In addition, research efforts in biohybrid robotics will likely have an important and immediate impact on tissue engineering and regenerative medicine (e.g., due to the optimization of upscaling techniques to build 3D tissues), as well as on in vitro muscle models for drug testing. The long-term impact of biohybrid actuators may be pervasive and global, because they may enable (i) soft robotic artifacts to safely interact with humans through lifelike movements, (ii) microscale devices to safely and extensively perform medical procedures, (iii) biobased surveillance systems, (iv) emerging manufacturing systems capable of self-assembly and self-repair, and (v) swarm biorobots for environmental monitoring, to face natural disasters or pollution, and other examples of biobots and living machines. Future robots are expected to be robust, flexible, and adaptive; they should learn from their experience and possibly self-repair. Future desirable tasks include, for example, autonomous exploration of inhospitable and unexplored territories and participation in search and rescue actions. Innovative actuators are strongly needed to achieve these performances, and biohybrid ones represent an exciting opportunity. Furthermore, the new knowledge generated by developing advanced biohybrid actuators will probably affect other scientific and technological fields that may deploy, in the future, an increasing number of biohybrid paradigms. This may concern not only actuation but also sensing and energy generation/storage.

SUPPLEMENTARY MATERIALS

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Movie S1. Biohybrid actuators based on bacteria and other motile cells.
Movie S2. Biohybrid actuators based on explanted whole-muscle tissues.
Movie S3. Biohybrid actuators powered by cardiomyocytes.
Movie S4. Biohybrid actuators based on insect-derived self-contractile tissues.
Movie S5. Biohybrid actuators based on engineered skeletal muscle.

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