ADVANCED REVIEW

Biohybrid robotics: From the nanoscale to the macroscale

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Abstract

Biohybrid robotics is a field in which biological entities are combined with artificial materials in order to obtain improved performance or features that are difficult to mimic with hand-made materials. Three main level of integration can be envisioned depending on the complexity of the biological entity, ranging from the nanoscale to the macroscale. At the nanoscale, enzymes that catalyze biocompatible reactions can be used as power sources for self-propelled nanoparticles of different geometries and compositions, obtaining rather interesting active matter systems that acquire importance in the biomedical field as drug delivery systems. At the microscale, single enzymes are substituted by complete cells, such as bacteria or spermatozoa, whose self-propelling capabilities can be used to transport cargo and can also be used as drug delivery systems, for in vitro fertilization practices or for biofilm removal. Finally, at the macroscale, the combinations of millions of cells forming tissues can be used to power biorobotic devices or bioactuators by using muscle cells. Both cardiac and skeletal muscle tissue have been part of remarkable examples of untethered biorobots that can crawl or swim due to the contractions of the tis-sue and current developments aim at the integration of several types of tissue to obtain more realistic biomimetic devices, which could lead to the next generation of hybrid robotics. Tethered bioactuators, however, result in excellent candidates for tissue models for drug screening purposes or the study of muscle myopathies due to their three-dimensional architecture.

KEYWORDS: bacteria-bots, biorobots, enzymatic nanomotors, hybrid robotics, muscle-based biorobots

1. INTRODUCTION

Biomimetics has always fascinated human beings, driving scientific knowledge and technological development to its utmost limits. We can find one of the first exponents of this enthusiasm in the works of Leonardo da Vinci, who observed the dynamics and the anatomy of flying birds and mammals, and composed designs of flying machines, resembling the structure of animal's wings that, unfortunately, never succeeding at flying. Half a millennium later, we still use the prescient view of Leonardo da Vinci and look at nature in a biomimetic manner to imitate features from biological systems that have been optimized for thousands and millions of years. From the adhesion of frogs and geckos (Bhushan, 2009) or the structural coloration of butterflies (Burg & Parnell, 2018) to the nanostructuring of stronger and tougher materials that mimic spider webs (Gabara, 2016) or the design of sustainable buildings that resemble termite mounds (Biomimicry Institute, 2019), biomimetics has found applications in virtually all fields of scientific research and technology. In the robotics field, human biomimicry has resulted in better prostheses and orthoses for impaired people (Cianchetti et al., 2018), as well as humanoid robots that replicate human expressions and facial features, imitate the range of movements of muscles and articulations or sense and respond to complex stimuli (Greshko, 2019; Markoff, 2013). However, these developments are still lagging far behind nature in terms of efficiency, robustness, control, energy storage, and power-to-weight ratio, among many other interesting characteristics, like self-healing or adaptation to complex stimuli (Carlsen & Sitti, 2014; Ricotti et al., 2017; Ricotti & Menciassi, 2012).

Engineering systems that can recapitulate some of the features of living organisms has been one of the challenges scientists and engineers in the last decades. One strategy has been the combination of biological entities with synthetic materials leading to the so-called “biohybrid robotics” field. These systems can span across different length-scales and have the advantage of combining the best of the “two worlds,” synthetic and biological, in a synergistic manner (Ricotti et al., 2017). Developments in biohybrid devices span all lengths scales, from the nanoscale to the macroscale. At the nanoscale, fundamental research in active matter and molecular biotechnology has led to the fabrication of biohybrid self-propelled nanoparticles powered by enzymatic reactions (Patiño, Arqué, et al., 2018). This type of devices has offered benefits in the creation of smart drug delivery systems that can reach larger distances and use biocompatible reactions as their power source. At the microscale, the use of biomolecules can be substituted by whole cells, such as bacteria or spermatozoa, to fabricate biohybrid microswimmers that utilize the complex and efficient propelling mechanisms of single cells to carry microparticles or microtubes, with applications in biofilm removal, drug delivery, cell manipulation or assisted reproduction (Carlsen & Sitti, 2014). Multiple cells in the form of tissues can also be applied for biohybrid robotic systems. In this case, muscle tissue, either skeletal or cardiac, is the choice par excellence in the field, as the contraction capabilities of this tissue can be used to power biorobots or bioactuators, biomimicking the structure and organization of the musculoskeletal system. Moreover, current developments aim at integrating several kinds of cells to create multicellular engineered living systems with complex and novel functionalities embedded in their design (Kamm et al., 2018).

Biohybrid research is continuously increasing (Figure 1). A bibliometric search shows that number of publications related to this topic has been increasing since 2005, with more than 25% of them being published between 2016 and today (Figure 1a). While most of these publications are of the article type, there have been an important number of reviews dealing with this subject and even some patents (Figure 1b). This search also demonstrates the large multidisciplinarity of the field,

with most of the contributions coming from engineering, cell biology, material science, chemistry, instrumentation, molecular biology or physics, while computer science and robotics occupy only the 10th and 11th positions, respectively (Figure 1c). Given the wide range of research fields and applicability, there is a need to find proper divisions and categories that can unify the nomenclature and the language used to describe these devices. Recent reviews in the literature have suggested approaches such as the macrocategorization in (i) application-oriented nonscalable devices, and (ii) general-purpose scalable devices (Ricotti et al., 2017). While the first category refers to a top-down approach, in which artificial technologies are built around an already optimized biological entity, such as bacteria-based microswimmers, the second category covers bottom-up strategies in which biological components, like single cells, are bioengineered together to form general-purpose large-scale biorobots, such as muscle-powered devices. Other approaches for categorizing existing biohybrid systems is the so-called robotic taxonomic key (RTK) (Webster-Wood et al., 2017). The RTK divides each system into four key elements: (i) structure, (ii) actuation, (iii) sensing, and (iv) control. Each of these elements can be either organic, hybrid or synthetic, and the final result offers a visual and convenient method for categorizing biohybrid robots. While both of these types of categorizations are valid, there is still lack of consensus in the literature regarding the proper nomenclature, or even what can be considered a biohybrid device. This focus review, however, does not aim at classifying the devices reported in the literature, but rather reviewing the type of biological integration at different length scales from a simpler point of view: from biomolecules at the nanoscale and microscale, single cells at the microscale, to eventually multicellular tissues at the macroscale.

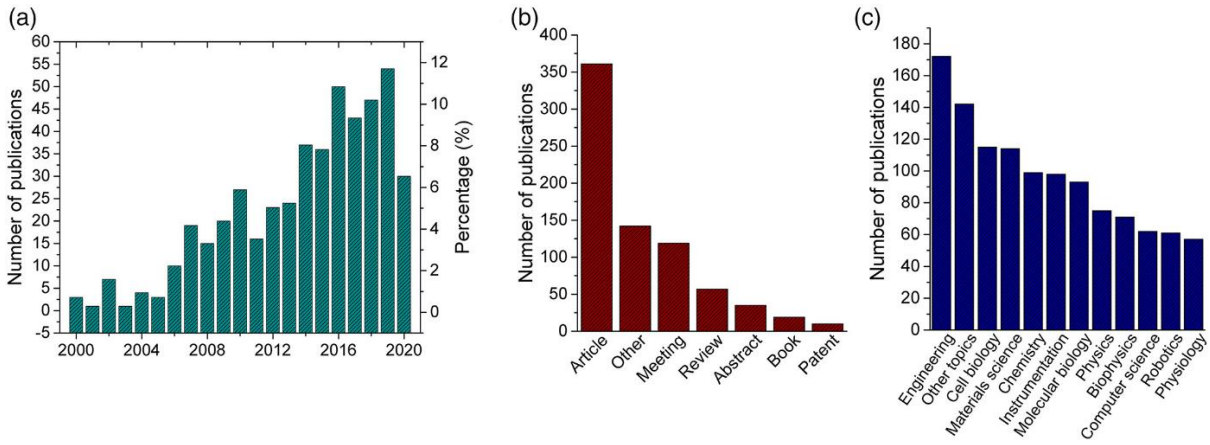

FIGURE 1
Status of biohybrid research in the literature, as of June, 2020. (a) Number of papers and percentage published between 2000 and June, 2020 on biohybrid robots. The analysis was performed in the Web of Science database searching for the following keywords in their title or abstract: “bio-hybrid actuator*,” “biohybrid actuator*,” “cell-based actuator*,” “bacteria robot*,” “muscle bioactuator*,” “muscle-based bioactuator*,” “muscle-based bio-actuator*,” “bioactuator*,” “bioactuator*,” “biohybrid robot*,” “bio-hybrid robot*,” “hybrid bio-robot*,” “hybrid biorobot*,” “bio bot*,” “bio-hybrid device*,” “biohybrid device*,” “bio-hybrid system*,” “biohybrid system*.” (b) Number of publications on biohybrid robots according to their type (Note: Several publications could have duplicate types). The search was performed using the same keywords. (c) Number of publications on biohybrid robots according to their field. The search was performed using the same keywords

2. HYBRID MACHINES AT THE NANOSCALE

2.1 Enzyme-powered nanomotors

At the nanoscale, hybrid machines that combine an artificial core structure with a biomolecular component to provide self-propulsion have been developed. These nanoparticles, also called nanomotors or nanoswimmers, found their earliest inspiration in the active transport in cells conducted by molecular motors (Hess & Vogel, 2001). Kinesins or myosins, for instance, are superfamilies of proteins known as motor proteins that can be found in eukaryotic cells and can generate forces by the hydrolyzation of adenosine triphosphate (ATP) to form adenosine diphosphate (ADP). Thanks to this, kinesins are able to move along microtubule filaments to support cellular functions and certain myosins are responsible for muscle contraction by sliding in actin filaments. This conversion of chemical energy into mechanical work, highly optimized through natural evolution, gave inspiration for the creation of nanomotors and micromotors that could move based on the same principles. One of the pioneer works was in 2002 by Whitesides et al., who presented millimeter-sized hemicylindrical plates that could self-propel by the decomposition of hydrogen peroxide by a Pt layer (Ismagilov et al., 2002). However, the first example of a motor at the nanoscale based on catalytic reactions was demonstrated in 2004 by Paxton et al., who used bimetallic Pt–Au nanorods to produce motion thanks to the same decomposition of hydrogen peroxide by Pt (Paxton et al., 2004). Shortly after, Fournier-Bidoz et al. also presented Ni/Au nanomotors based on catalytic reactions, but this time peroxide decomposition was achieved in the Ni part (Fournier-Bidoz et al., 2005). Since then, nanomotors with a wide variety of sizes and geometries have been demonstrated, for the most part based on chemical reactions catalyzed by different metals, finding their most interesting applications in fundamental physics (Katuri et al., 2017; Romanczuk et al., 2012) or environment applications (Parmar et al., 2018; Vilela et al., 2017).

The first biohybrid swimmer based on enzymes was also millimeter-sized. Mano and Heller designed and fabricated a bioelectrochemical system composed of a carbon fiber with two enzymes at each side, glucose oxidase (GOx) and bilirubin oxidase (BOD), which resulted in a net power-generating reaction that could propel the fiber on a water-air interface (Mano & Heller, 2005). Later on, Pantarotto et al. fabricated the first nanomotor based on the tandem reaction of GOx and catalase that could move by the catalytic conversion of glucose into D-glucono-1,5-lactone and hydrogen peroxide by GOx and the subsequent decomposition of hydrogen peroxide by catalase (Pantarotto et al., 2008). However, several issues affected the motion of these enzyme-based motors, such as movement only taking place at the air-liquid interface in the first case or the need of a continuous flow of pure oxygen to ensure the activity of GOx in the second case. Afterwards, research toward the development of more energy efficient, versatile and biocompatible hybrid nanomachines based on enzymes increased exponentially (Ma, Hortelao, et al., 2016; Patiño, Arqué, et al., 2018; Sanchez et al., 2010; Y. Wang et al., 2006).

Nanomotors and micromotors based on inorganic catalysts

After the first demonstrations of catalytic nanomotors using bimetallic Pt–Au (Paxton et al., 2004) and Ni-Au (Fournier-Bidoz et al., 2005) nanorods, the field of self-propelled nanoparticles and microparticles has received increasing attention over the last years (Katuri et al., 2017; Sanchez et al., 2015). Apart from enzymes, metallic catalysts, especially Pt, have been the most common type of power source, although others have used light (Palagi et al., 2017; L. Xu, Mou, et al., 2017; Xuan et al., 2018) or ultrasound waves (T. Xu, Gao, et al., 2017). Artificial microjets, fabricated by roll-up nanotechnology (Mei et al., 2008, 2011) or electrodeposition with porous membranes (Gao et al., 2011; Zhao & Pumera, 2013), can move through bubble propulsion when a metallic catalyst is present in their interior and surfactant in the surrounding environment (Simmchen et al., 2014; Solovev et al., 2009). These jets usually move at high speeds and show their most promising applications in industrial cleaning of polluted water (Guix et al., 2012; Parmar et al., 2018; Soler et al., 2013), but also in biosensing (Campuzano et al., 2011), drilling of soft matter (Solovev et al., 2012) and transport of cargo on-chip (Solovev et al., 2010; J. Wang, 2012). Spherical self-propelled microparticles and nanoparticles are usually achieved by the half-coating of their surface with a metallic element in the form of Janus particles (Howse et al., 2007). Usually, the degradation of H2O2 by the Pt cap creates phoretic fields that allow the particle to migrate with active motion subjected to Brownian fluctuations, but whether the motion mechanism relies on self-diffusiophoresis (Golestanian et al., 2005, 2007), self-electrophoresis (Brown & Poon, 2014; Ebbens et al., 2014) or a more complex interaction is still under debate. These Janus particles are excellent candidates for fundamental studies in active matter due to their ease of fabrication and characterization (Bechinger et al., 2016; Ebbens et al., 2012; Ginot et al., 2018; Howse et al., 2007; Illien et al., 2017). Interesting behaviors have been demonstrated, such as cross-stream migration in flows (Katuri, Uspal, et al., 2018) or steering with surfaces (Das et al., 2015; Katuri, Caballero, et al., 2018; Uspal et al., 2015), which could be used to pick up cargo (Baraban et al., 2012) or actuate microgears (Maggi et al., 2015). However, their applications in biomedicine are limited due to the use of H2O2 for their propulsion and enzymes come as a more preferable choice for biocompatibility reasons.

The combinations of enzymes, geometries, sizes and motion mechanisms are virtually endless (Patiño, Arqué, et al., 2018) (Figure 2). Regarding size, smaller hybrid nanomotors based on the half-functionalization of silica nanoparticles of 90 nm with catalase (creating the so-called Janus particles) can achieve motion by enhanced diffusion (Figure 2a) (Ma & Sánchez, 2017), while on the other end, hollow silica microparticles of 2 μm of diameter heterogeneously coated with urease can propel themselves with directionality (Ma, Wang, et al., 2016). Their geometries can range from spherical particles, as in the previous cases, to tubular shapes that can be propelled by bubble generation when their dimensions are micron-sized (Sanchez et al., 2010) or bubble-free propulsion if they are nanotubes (Figure 2b) (Ma, Hortelao, et al., 2016). A few different kinds of enzymes have also been employed. Ma et al. fabricated Janus nanomotors based on silica nanoparticles using three types of enzymes: urease, GOx and catalase (Ma et al., 2015). Due to the induced asymmetry of the enzyme functionalization, all types of motors showed enhanced diffusion after the addition of their corresponding fuels, namely urea, glucose and hydrogen peroxide. Later, they proved the controllability of urease-powered nanomotors by the addition of urease inhibitors (Ag+ and Hg2+) to stop the motion and dithiothreitol (DTT) to reactivate it by competitive binding (Ma, Wang, et al., 2016). Moreover, the deposition of a thin Fe layer allowed the guidance of these biohybrid nanomotors with magnetic fields. Other geometries and materials have also been studied, such as the polymeric stomatocytes by Abdelmohsen et al., who

encapsulated catalase and GOx enzymes inside them, achieving motion of these nanomotors while protecting the enzymes from the external influences of the media (Figure 2c) (Abdelmohsen et al., 2016), and liposomal vesicles (also called Lipobots) that can carry urease enzymes either inside or outside their lipidic layers and can enhanced their motility by the addition of sodium deoxycholate, which modifies the fluidity of the lipid bilayer (Figure 2d) (Hortelao, García-Jimeno, et al., 2020).

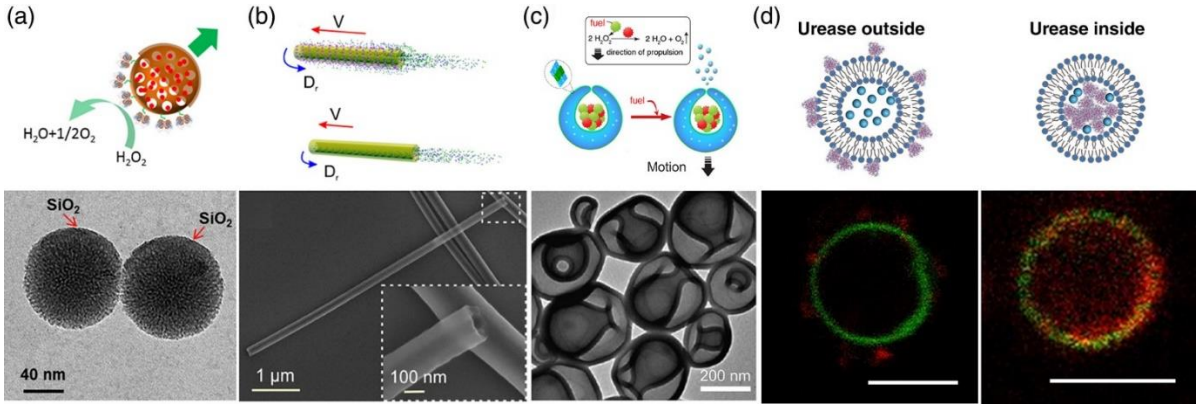

FIGURE 2
Biohybrid nanomotors powered by enzymatic reactions. (a) Top: Schematic of the structure and working mechanism of a biocatalytic Janus mesoporous silica nanomotor. Bottom: Transmission electron microscope (TEM) image of the mesoporous silica nanoparticles that compose such motor. Adapted with permission from Ma and Sánchez (2017). (b) Top: Schematic representation of the motion of urease-powered nanotubes depending on enzyme localization: Inside and outside (up) or only inside (bottom). Bottom: Scanning electron microscope (SEM) image of the silica nanotubes. Adapted with permission from Ma, Hortelao, et al. (2016). (c) Top: Schematic representation of the structure and working mechanism of a polymeric stomatocyte nanomotor with multiple enzymes encapsulated in its interior and working via cascade decomposition of glucose and hydrogen peroxide by GOx and catalase. Bottom: TEM image of a group of polymeric stomatocytes used to fabricate this nanomotor. Adapted with permission from Abdelmohsen et al. (2016). (d) Top: Schematic representation of lipobots with urease molecules outside or inside the lipidic layer. Bottom: Confocal laser scanning microscopy images of urease (red) in micrometer-sized lipobots (green) with the enzyme located outside or inside. Scale bars are 2 µm. Adapted with permission from Hortelao, García-Jimeno, et al. (2020)

Most of the applications of hybrid nanomotors have aimed at the development smart drug delivery (J. Wang & Gao, 2012) or medical imaging systems (van Moolenbroek et al., 2020). For instance, Ramos-Docampo et al. used polystyrene nanomotors functionalized with collagenase to improve the internalization on cell spheroids by the cleavage of collagen by the enzyme (Ramos-Docampo et al., 2019). They showed that calcium ions could be used as an activator, displaying an enhanced internalization only when calcium was present. GOx-catalase nanomotors triggered by near infrared light (NIR) were shown to improve drug delivery in vitro to cancer cells through synergistic photodynamic and starvation therapies (You et al., 2019). Enhanced diffusion of urease-powered nanomotors can improve the efficiency of anticancer drugs in 2D and 3D cultures of bladder cancer cells by showing an increased internalization when the nanomotors are targeted to a transmembrane protein (Figure 3a) (Hortelao et al., 2019; Hortelão et al., 2018). The same system was recently used in combination with the isotopes Iodine-124 and Fluorine-18 to study their suitability for in vitro and in vivo imaging via positron emission tomography (PET) coupled with computed tomography (PET-CT) (Hortelao, Simó, et al., 2020), following a previous study that showed the successful imaging of motors of micrometer scale by chemisorption of Iodine into their gold surface (Vilela et al., 2018). Upon addition of urease, the nanomotors presented complex swarming behavior that could be tracked by PET-CT (Figure 3b) and were homogeneously

distributed in the bladder cavity of mice after intravesical administration (Figure 3c) (Hortelao, Simó, et al., 2020).

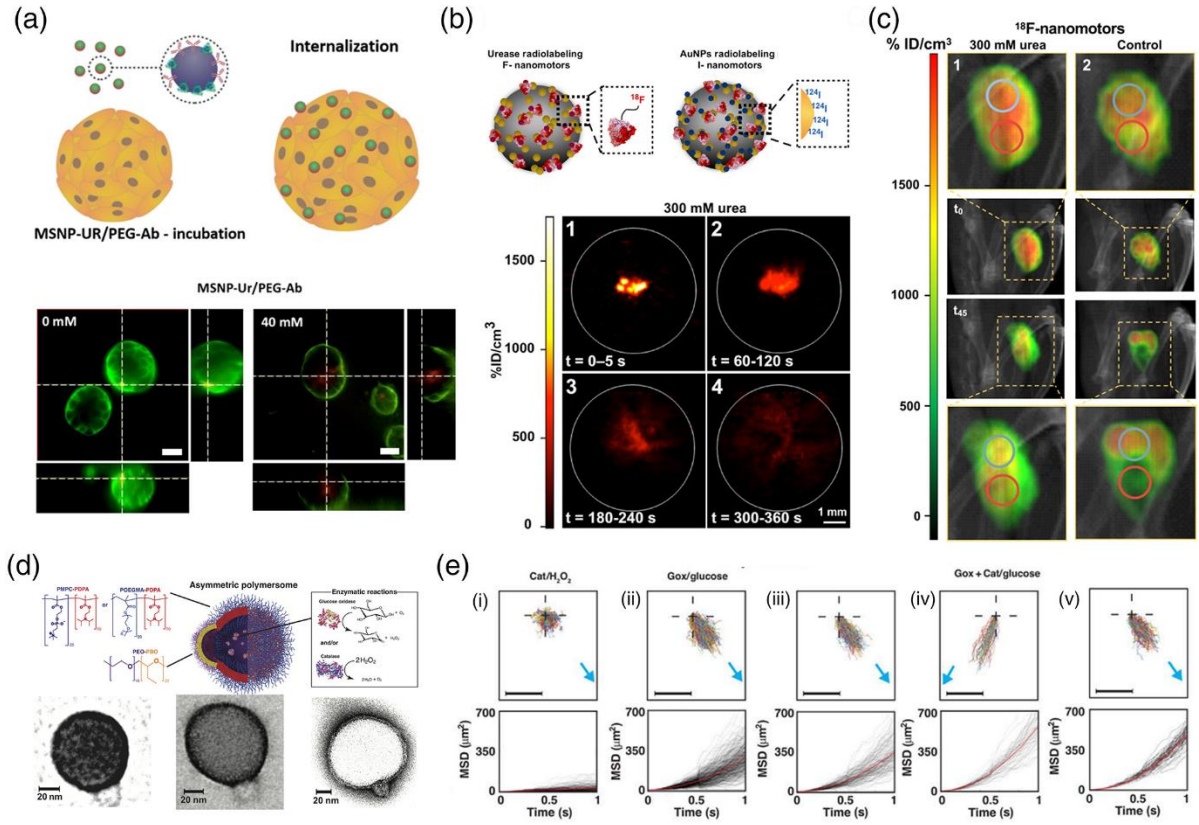

FIGURE 3
Applications of biohybrid nanomotors powered by enzymatic reactions. (a) Schematic representation of a urease-powered silica nanomotor functionalized with antibodies for enhanced penetration in bladder cancer spheroids. Confocal fluorescence images of nanomotors incubated with spheroids at 0 and 40 mM of urea (scale bars are 50 μm). Green: targeted antigen (FGFR3); red: nanomotors. Adapted with permission from Hortelao et al. (2019). (b) Schematic representation of urease-powered nanomotors radiolabeled with Fluorine-18 and Iodine-124 (attached to gold nanoparticles). Bottom images show swarming behavior of these nanomotors after the addition of urea via PET in vitro. Adapted with permission from Hortelao, Simó, et al. (2020). (c) in vivo PET-CT images of urease nanomotors radiolabeled with Fluodine-18 after intravesicular administration, with and without urea, showing their distribution in the bladder of mice. Adapted with permission from Hortelao, Simó, et al. (2020). (d) Schematic representation of an asymmetric polymerosome nanomotor propelled by the tandem reaction of GOx and catalase. Bottom images show characterizations of the polymerosome imaged under several types of staining targeting different polymers. Adapted with permission from Joseph et al. (2017). (e) Normalized trajectories and corresponding mean squared displacements (MSDs) of asymmetric polymerosomes under different conditions of fuels and directions. Adapted with permission from Joseph et al. (2017)

However, before more studies can be carried out in vivo, biological barriers such as interstitial flow pressure, phagocytic sequestration or endosomal escape need to be investigated, as they pose challenges for the use of these devices in clinical trials (Blanco et al., 2015). For this purpose, approaches based on enzyme cascade reactions or compound systems with various functionalizations, such as peptides, antibodies or light-responsive moieties are being developed. For instance, Joseph et al. recently showed the fabrication and use of asymmetric polymerosomes with tandem GOx-catalase reactions that could follow gradients of glucose (Figure 3d,e). By performing in vivo studies, they found an almost 4-fold increase of nanomotor delivery through the blood brain barrier in rats (Joseph et al., 2017). Other systems based on chemotaxis, cascade reactions, compartmentalization, gatekeepers for on-demand release of drugs or particle

functionalization with several moieties that fulfill different purposes, are being investigated to safely and efficiently use these devices as drug delivery methods (Abdelmohsen et al., 2016; Llopis-Lorente et al., 2019; Nijemeisland et al., 2016; Peng et al., 2017; Schattling et al., 2017; Y. Wang, Cui, et al., 2017; Y. Wu et al., 2014; Zhao et al., 2018).

Despite all the advances in drug delivery applications, there is a growing need for a better understanding of the dynamics of this type of nanomotors, their interactions with complex media or their response to certain stimuli. For instance, the effects of salts or other molecules present in physiological fluids are not completely understood, as they could affect the reactions catalyzed by the enzymes, and thus their movement (De Corato et al., 2020; Hortelao et al., 2019). Their motion in simple Newtonian fluids like water, although necessary to characterize and optimize their performance, is not an appropriate model of an in vivo environment, since the nanomotors will need to interact with the crowded environment of tissues and cells, affecting their diffusion and biocatalytic reactions. More investigation of the motion of nanomotors in complex environments will be necessary to optimize their fabrication to move efficiently in biological fluids or protect them from adverse effects (Palagi et al., 2017).

3. HYBRID MACHINES AT THE MICROSCALE

3.1 Enzyme-powered micromotors

At the microscale, Brownian fluctuations stop playing a fundamental role in motion of hybrid micromachines and viscosity becomes an essential aspect of their movement. As it was described by Purcell in 1977, motion in a fluid at the microscale, or low Reynolds number, is dominated by the viscous properties of the liquid and time-irreversible mechanisms are necessary to achieve net displacements (Purcell, 1977). Enzymatically propelled micromotors can move at this scale, as they have a continuous source of energy coming from biocatalytic reactions. In biomedicine, enzymatic micromotors are of interest since their propulsive motion can increase the explored area and their driving force, which ranges from tens of fN at the nanoscale (Ma et al., 2015) to hundreds of fN at the microscale (Patino et al., 2019; Patiño, Feiner-Gracia, et al., 2018), enhancing the possibility of penetrating tissues. Besides their applications in biomedicine, these micromotors are also crucial in fundamental applications trying to decipher their underlying mechanisms of motion, as their bigger size allows them to be characterized more easily. At the nanoscale, enzymatic nanomotors show enhanced diffusion due to the effect of Brownian fluctuations but, at the microscale, it is possible to achieve directionality and ballistic motion, which can help in the extraction of parameters that characterize their movement (Howse et al., 2007). As their underlying mechanisms of motion are still to be completely defined due to the heterogeneity of the systems, current analysis methods are mainly based on the mean square displacement (MSD) of the particles' trajectory for the extraction of parameters, which is independent of the motion mechanism. However, complex motion behaviors might not be accurately described by the classical equations to describe their movement and more robust or generalized approaches are being studied (Mestre, Palacios, et al., 2020).

It is generally assumed that an asymmetry in enzyme coverage in the form of a Janus particle is necessary for net motion to happen (Golestanian et al., 2007; Ma et al., 2015; Ma & Sánchez, 2017). However, it was demonstrated using micron-sized urease-powered motors that full functionalization of a particle's surface with urease can result in active motion due to

inhomogeneous or patchy-like coverage (Figure 4a,b) (Patiño, Feiner-Gracia, et al., 2018). Motion analysis and optical tweezer measurements revealed that the amount of urease molecules needed to achieve motion displayed a minimum threshold below which only Brownian diffusion was observed (Figure 4c). Intrinsic enzymatic properties can also affect the motion of the hybrid micromotors. Arqué et al. studied the catalytic rate and conformation changes during catalysis to understand their contribution to active motion through optical recordings and molecular dynamic simulations, using four different enzymes: urease, acetylcholinesterase, GOx, and aldolase (Arqué et al., 2019). It was found that high catalytic activity was crucial for efficient motion and that conformational dynamics were required for certain biocatalyzes to take place (Figure 4d). On this topic, the orientation of the enzyme when immobilized to a particle's surface was found to play a key role in its motility, demonstrating that hydrophobic adsorption of lipase in nanomotors led to the most efficient catalytic process (L. Wang et al., 2020).

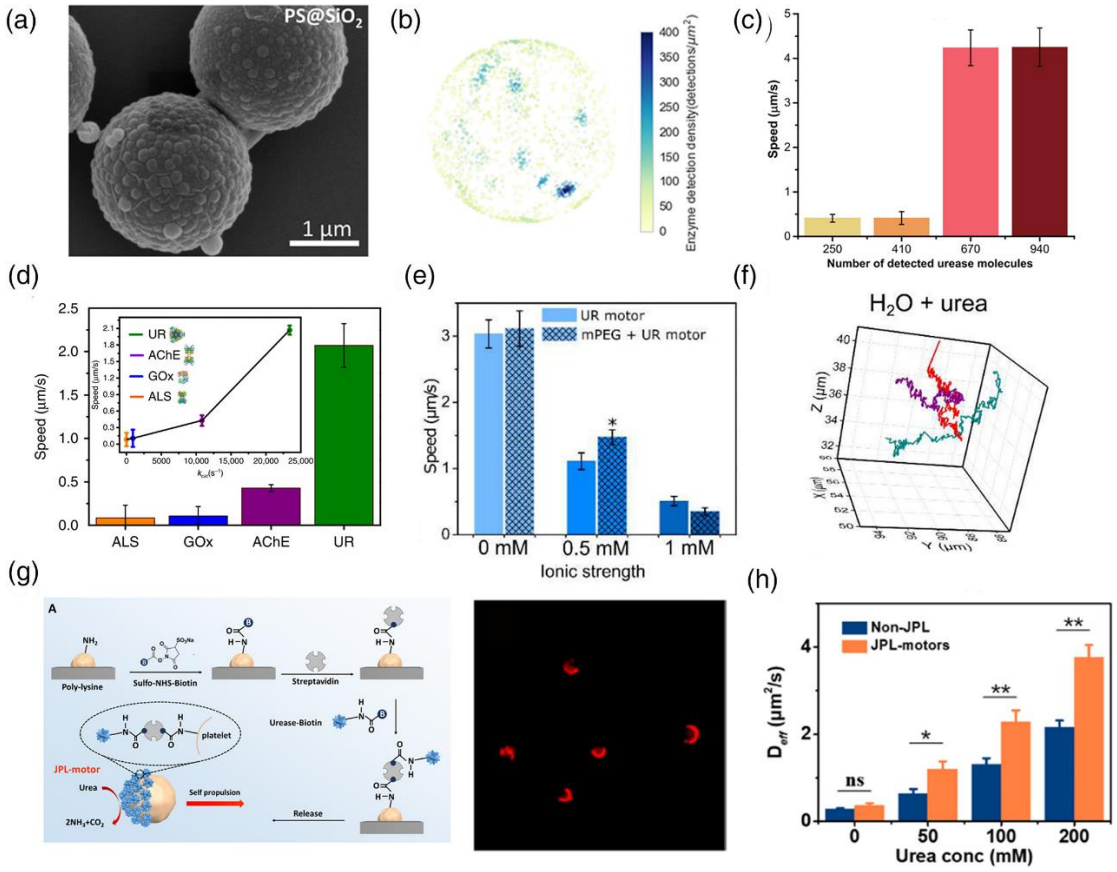

FIGURE 4
Applications of enzyme-powered micromotors. (a) SEM image of silica microparticles used to fabricate urease-powered micromotors. Adapted with permission from Patiño, Feiner-Gracia, et al. (2018). (b) Computer-generated representation of the location of the heterogeneous localization of enzymes, obtained with super resolution microscopy. Adapted with permission from Patiño, Feiner-Gracia, et al. (2018). (c) Speed of the urease-powered micromotors depending on the amount of detected enzyme molecules on its surface. Adapted with permission from Patiño, Feiner-Gracia, et al. (2018). (d) Average speed of biohybrid micromotors based on hollow silica microparticles depending on the enzyme used as biocatalytic engine. Inset shows the correlation of the speed with the catalytic rate of the enzyme. Adapted with permission from Arqué et al. (2019). (e) Effect of the ionic strength of NaOH in the speed of these micromotors with or without an mPEG coverage. Adapted with permission from Arqué et al. (2020). (f) 3D trajectory mapping of urease-powered micromotors showing directionality in all directions. Adapted with permission from Arqué et al. (2020). (g) Schematic fabrication of the Janus platelet motors powered by urease. Right: Fluorescent images in Cy5-labeled urease showing the Janus functionalization of the motors. Adapted with permission from Tang et al. (2020). (h) Enhanced diffusion of Janus and non-Janus motors after the addition of urea. Adapted with permission from Tang et al. (2020)

Ionic species and pH can also affect the motion of enzymatic motors. Self-sensing urease-based micromotors based on DNA nanoswitches showed a decrease in motility after the release of ammonia upon decomposition of urea and subsequent change of pH (Patino et al., 2019). Furthermore, the study of enzyme-powered microparticles in ionic solutions helped determine the role of ionic species and Debye length in their motion with numerical simulations, finding an inverse relationship between micromotor speed and the background electrolyte concentration that matched experimental values (De Corato et al., 2020). Further tracking experiments in 2D and 3D of urease-powered micromotors under different pH and ionic strengths showed a reduction of their self-propulsion that pointed toward ion-dependent mechanism of motion (Figure 4e,f) (Arqué et al., 2020). Moreover, methoxypolyethylene glycol amine (mPEG) was used to mitigate the effects of the ionic species, obtaining a small recovery in the speed after increasing the micromotor Debye length. Recently, Janus micromotors composed of platelets functionalized with urease molecules via biotin-streptavidin linkage showed enhanced diffusive motion, even under the presence of ionic species in PBS and biological fluids (Figure 4g,h) (Tang et al., 2020). All these diverse results highlight the importance of the surface chemistry, surface charges and type of enzyme linkage in the micromotor design and fabrication, which might give rise to different relationships between ionic species and motion efficiency. In conclusion, enzymatic motors at the microscale pose interesting examples of active matter systems and are relevant tools in fundamental research aiming at understanding their intricate motion mechanisms and the limitations imposed by the presence of ions in the media need to be overcome for an efficient implementation in biomedical applications.

3.2 Single-cell-powered microswimmers

Beyond enzymatic micromotors, we can find at the microscale further examples of biological motility or activity in microorganisms. Some single cells have developed sophisticated mechanisms to achieve motion at low Reynolds number with time-irreversible strokes. For example, bacterial species, such as Escherichia coli, Serratia marcescens or magnetotactic bacteria, sperm cells or even strains of protozoa or algae can generate forces for motion thanks to flagella or cilia differently distributed along their bodies (Carlsen & Sitti, 2014; Magdanz et al., 2013; O. Yasa et al., 2018). The incorporation of these single-cell organisms by their entrapment in microtubes or their attachment to microparticles leads to the fabrication of biohybrid microswimmers that use the motion capabilities of these cells for their advantage (Figure 5). The use of these organisms also entail other types of benefits, such as their ease of acquisition, their survival under rough environmental conditions, like high temperature or acidic pH, and the requirement of low amount of simple nutrients, like glucose, for their survival (Carlsen & Sitti, 2014). Moreover, secondary control mechanisms, such as magnetotaxis, chemotaxis, galvanotaxis, phototaxis, thermotaxis, or aerotaxis can be used to direct the motion of these types of hybrid robots toward specific directions (Martel, 2012; Ricotti et al., 2017). Different types of applications have been envisioned, ranging from drug delivery systems (B. W. Park et al., 2017; H. Xu et al., 2018; O. Yasa et al., 2018) and fertilization methods (Striggow et al., 2020) to bacteria biofilm removal (Stanton et al., 2017) and microscale manipulation (Angelani et al., 2009; Di Leonardo et al., 2010; Martel et al., 2006).

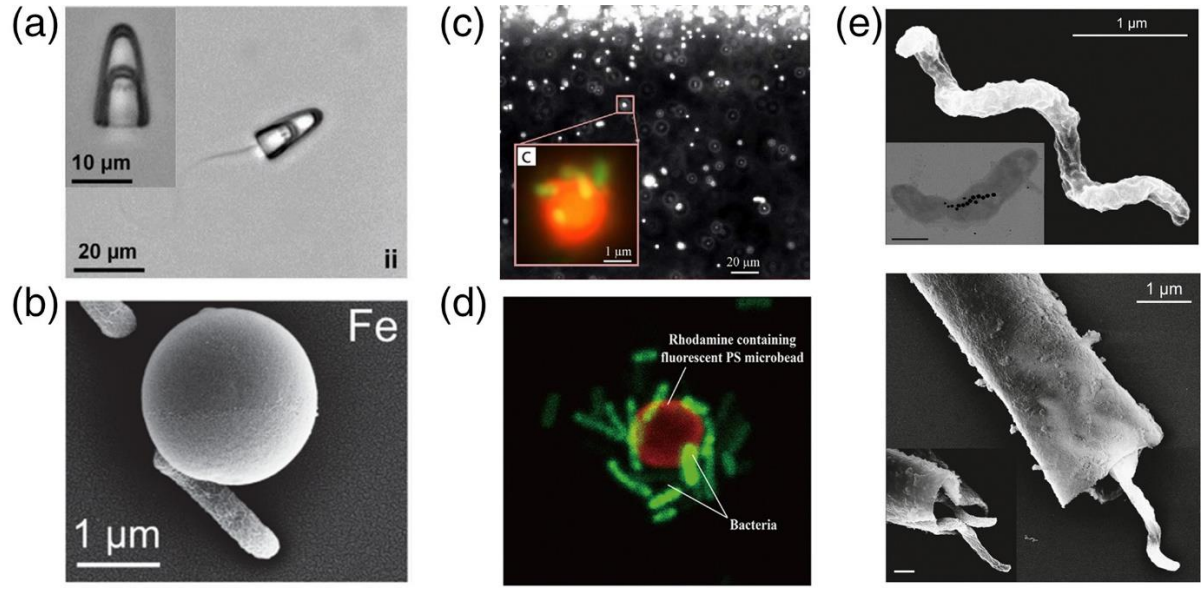

FIGURE 5
Biohybrid microrobots based on single cells. (a) Optical microscopy image of a sperm-driven microrobot with a streamlined cap design. Adapted with permission from Striggow et al. (2020). (b) SEM image of a biohybrid microrobot based on Escherichia coli. The bacterium attaches preferentially to the metallic Fe surface of the microparticle, deposited by e-beam. Adapted with permission from Stanton et al. (2016). (c) Fluorescent image of a swarm of bacteria-based microswimmers moving by chemotaxis toward the upper side of the image. Inset shows a fluorescently labeled image of a microswimmer, composed of a polystyrene bead and several S. marcescens attached in random positions. Adapted with permission from Zhuang and Sitti (2016). (d) Fluorescent confocal microscopy image of several Salmonella typhimurium bacteria (green) attached to polystyrene beads (red) to form a bacteriobot. Adapted with permission from S. J. Park et al. (2013). (e) (Top) SEM image of a nonpathogenic magnetotactic bacterium Magnetosopirrillum gryphiswalense (MSR-1), with an inset of TEM image showing the distribution of the internal magnetosome. (Bottom) SEM image of an MSR-1 bacterium captured within a microtube, forming a biohybrid micromotor. Adapted with permission from Stanton et al. (2017)

The use of spermatozoa can result in interesting applications in artificial fertilization techniques, as well as in fundamental aspects of the motion of these single-cell organisms. For instance, a biohybrid sperm-bot was fabricated by Magdanz et al. by encapsulating sperm cells in a rolled-up microtube consisting of Ti and iron that could be controlled via magnetic fields, offering a promising system for alternative fertilization methods (Magdanz et al., 2013). Likewise, in another publication, sperm cells were trapped into microtubes and their motion in simulated viscoelastic oviduct fluid was demonstrated, as well as their guidance using magnetic fields (Figure 5a) (Striggow et al., 2020). Moreover, the heads of dead sperm cells were coated with magnetic nanoparticles and stimulated with rotating magnetic fields, simulating the rotatory motion of the sperm's flagella. The authors studied the propagation of waves of magnetically actuated sperm cells and compared their thrust forces and speed with alive cells, providing insights into the efficient motion of spermatozoa at low Reynolds number (Khalil et al., 2020).

Generally, bacterial organisms have been used to propel beads (Edwards et al., 2013; D. Kim et al., 2012; Zhuang & Sitti, 2016), microtubes (Stanton et al., 2017) or even microgears to convert their chemical energy into mechanical work (Angelani et al., 2009; Di Leonardo et al., 2010). As bacteria is attracted to metal surfaces, the integration of these organisms with metallic microparticles are in general straightforward. Stanton et al. demonstrated the fabrication of biohybrid Janus microswimmers driven by E. coli, using e-beam deposition to cover half of the surface of polystyrene microparticles with Ti, Au, Fe, or Pt (Figure 5b) (Stanton et al., 2016). Single bacteria readily attached themselves to the metallic surface of the particles and transported them with

magnetic field guidance. Later, Singh et al. developed an engineered method to improve the adhesion of these cells into microparticles by biotin-streptavidin linkages to create Janus-like hybrid particles, greatly improving the stability of the attachment when compared to Au, Pt, or glycol monolayers (Singh & Sitti, 2016). The increasing engineering complexity of these biohybrid systems has led to the development of multifunctional bacteria-driven microswimmers with embedded magnetic nanoparticles that could deliver an anticancer drug to cancerous cells in vitro with magnetic guidance, being proposed as the next generation of in vivo targeted drug delivery systems (B. W. Park et al., 2017).

The collective motion of multiple bacteria or biohybrid swimmers has also been investigated. Zhuang et al. studied the chemotactic drift toward L-serine of thousands of hybrid swimmers driven by multiple bacteria in microfluidic channels (Figure 5c) (Zhuang & Sitti, 2016). Likewise, green-fluorescent-protein-(GFP)-expressing E. coli were attached to spherical and disk-shaped microparticles and showed chemotaxis toward L-aspartic acid (Sahari et al., 2014). Another type of bacteria, S. typhimurium was used in conjugation with polystyrene beads via biotin-streptavidin linkage, forming bacteriobtos, to target and deliver microstructures to in vivo tumors thanks to their chemotactic behavior (Figure 5d) (S. J. Park et al., 2013). Later on, this same strain of bacteria was used by the same research group in a biohybrid device to investigate the effects of a chemorepellent, NiSO4 (D. Park et al., 2014). Other control mechanisms, such as pH-taxis, which could be useful in cancer treatment applications, have also been demonstrated (Zhuang et al., 2015). Likewise, magneto-aerotactic bacteria have already transported drug-loaded liposomes into hypoxic regions of tumors in mice (Felfoul et al., 2016). All of these examples prove the great versatility of these hybrid bacterial systems thanks to their environmental control methods, the possibility of genetically modifying them or the encapsulation of drugs, which could be exploited for targeted therapeutics (Hosseinidoust et al., 2016). However, several challenges, such as the toxicity of the bacterial cells, immune responses or the loss of engineered behavior are major concerns to be investigated and that might need response in a one-by-one basis. Nonetheless, several solutions, such as the use of the human microbiota or the tools offered by synthetic biology to delicately engineer bacteria strands, have been proposed (Hosseinidoust et al., 2016).

Magnetotactic bacteria are a particular kind of organisms that can achieve high swimming speeds and synthesize a chain of nanoparticles called magnetosomes, made of Fe3O4 crystals, that allow them to align to the Earth's magnetic field (Martel et al., 2009). Unlike many of the previous cases, which used artificial materials with magnetic properties to guide the direction of the biohybrid robot, this type of bacteria can be used as both the propulsion system and steering system, and have been proposed for MRI tracking in drug delivery applications (Martel et al., 2009). Other promising applications include biofilm removal, since these colonies are generally difficult to eliminate and resistant to antibiotic treatment, requiring more targeted approaches (Stanton et al., 2017). On this regard, Stanton et al. used magnetotactic bacteria to effectively target and reduce E. coli biofilms by releasing an antibiotic triggered by the acidic environment of the biofilm itself (Figure 5e) (Stanton et al., 2017).

Flagellar motion cannot only be achieved with organisms that have that kind of power source. With a biomimetic approach, Williams et al. fabricated a synthetic flagellar swimmer operating at low Reynolds number (Williams et al., 2014). This swimmer was made of polydimethylsiloxane (PDMS) filaments attached to a short, rigid head, resembling a sperm cell. In order to obtain an autonomous power source, they used single cardiomyocytes, the contractile cells that make up heart muscle tissue. By selectively functionalizing different parts of the swimmer, cardiac cells

were attached to the region near the head, producing sufficient power strokes that lead to powerful tail thrusts. These contractile cells, which contract spontaneously and synchronize with each other, generated motion of the biohybrid swimmer at approximately 10 µm/s.

Motion at the microscale can be difficult to achieve artificially due to the strong influence of viscous forces over inertial forces. However, in biohybrid cell-based robots, this issue can be less critical due to the use of the propelling mechanisms of bacteria or sperm cells, which have been optimized through evolution for millions of years. In these cases, most of the obstacles reside in their efficient assembly, their precise control and their potential toxicity for in vivo applications. The field of enzymatically powered motors at the microscale, on the other hand, faces several challenges regarding the description of their dynamics. Research in biohybrid motors at the nanoscale is primarily focused on biomedical applications and their challenges revolve around understanding their interaction with complex environments, such as the crowded interior of cells, extracellular matrices or full tissues. Micromotors, on the other hand, are often used as model systems for fundamental research into the parameters that affect their biocatalytic motion. Despite their bigger size and easier characterization, they are still lacking a complete description of their motion mechanisms and further research should focus on the generalization of the governing equations to embrace the complex motion that might arise from the increasing number of enzymatic micromotors being developed.

4. HYBRID MACHINES AT THE MACROSCALE

At the macroscale, ranging from hundreds of µm to several cm, the field biohybrid robotics has taken advantage of whole tissues to produce fully functional hybrid machines with outstanding characteristics adopted from biological systems. As it was briefly mentioned at the beginning, biomimetics has led to the creation of humanoid robots that can replicate many biological functions and ranges of movement. However, the performance of these machines is still far from the efficiency of natural systems, despite all the great efforts through biologically inspired engineering (Patino et al., 2016). In classic robotics, simple tasks for animals, such as the handling of fragile objects or moving through irregular or unknown terrains, remain difficult to achieve due to the rigidity of the materials used. Especially when these robots need to be used for human interaction or for medical operations, finding soft materials that can be integrated in these systems has become an important area of research (Ilievski et al., 2011). Soft robotics aims at exploiting, in a biomimetic way, the types of structures found in nonskeletal parts of animals or marine organisms, such as the tentacles of squids (Wehner et al., 2016), the muscle tissue of mammals (Chou & Hannaford, 1996) or the trunks of elephants (Martinez et al., 2013).

Artificial muscles

Material science research has led to the creation of different types of soft polymers that can be used as actuators that attempt to replicate muscle contraction. Electroactive polymers (EAPs), for instance, are electrically activated soft actuators that could have potential in the development of muscle-like actuators thanks to their capabilities of converting electrical energy into mechanical energy (Ji et al., 2019; Patino et al., 2016; Romasanta et al., 2015). EAPs can be divided according to their actuation method into ionic EAPs, driven by the mobility of ions, or electronic EAPs, driven by Coulomb interactions or electric fields (Romasanta et al., 2015), and many developments have been achieved in both types of actuators (Duduta et al., 2016; Opris, 2018; H. S. Wang, Cho, et al.,

2017). However, several limiting factors, such as their electrochemical performance, the need of prestretching or the high voltages and power density typically required for actuation hinder their universal applicability in soft robots (Duduta et al., 2016; Ji et al., 2019; Romasanta et al., 2015; H. S. Wang, Cho, et al., 2017). Pneumatically driven actuators or pneumatic artificial muscles (PAMs) are also among the most highly researched biomimetic actuators (Cho et al., 2020; Rus & Tolley, 2015). These actuators are composed of thin and flexible membranes reinforced with fibers that can be actuated with pressurized air (Martinez et al., 2013). The design of soft artificial muscles is usually based on agonist–antagonist pairs to better mimic the arrangement of skeletal muscle and achieve better compliance and bidirectional actuation. In the field of material science, research on smart polymers is advancing toward materials with the capabilities of sustaining high stresses, self-heal (Pena-Francesch et al., 2020) with programmable properties (Davidson et al., 2019), although there are still challenges to be tackled, such as their slow healing speed, low healing strength or difficulty in designing complex deformation profiles.

In particular, the replication of muscles has become one of the most important challenges in soft robotics, as they are a crucial component of the musculoskeletal system that allow animals to move with extreme sensitivity. However, the level of control, sensitivity and adaptability of skeletal muscle is difficult to replicate with artificial materials, although promising examples based on fluid-driven actuators have been developed. Martinez et al. developed robotic tentacles that could grasp objects in different ways using pneumatic soft actuators, and even embedded a video camera in them (Martinez et al., 2013). Fully soft and pneumatically driven robots (Shepherd et al., 2011; Tolley et al., 2014) or their combination with hard parts (Stokes et al., 2014) have also appeared as interesting examples toward applications in search and rescue missions. As classical materials for soft actuators reach their limitations, other types of actuation based on hybrid mechanisms are being developed. Hydraulically amplified self-healing electrostatic (HASEL) actuators can generate hydraulic pressure locally by electrostatic forces, taking advantage of the versatility of soft fluidic actuators with the self-healing properties of dielectric ones (Acome et al., 2018; Rothemund et al., 2020). Fully autonomous soft robots, however, are still far from being as efficient as animals, as electronic components or power sources are usually hard and heavy, and stretchable electronics, flexible sensors or complex control systems might be required (Rus & Tolley, 2015; Walker et al., 2020). Wehner et al. presented a fully untethered and autonomous robot made of soft materials, called “octobot,” that could move its tentacles thanks to microfluidic logic based on catalytic decomposition of hydrogen peroxide (Wehner et al., 2016). Despite its simple mode of actuation (raising and lowering its tentacles), this integrated design with all the necessary components for autonomous actuation might make it the foundation for the next generation of fully soft and autonomous robots.

It is not surprising to think that, due to great limitations of current muscle actuators, biohybrid devices have focused on the integration of muscle tissue from different sources into small robotic systems. The majority of biohybrid robots in the literature have used muscle from mammalian sources, either cardiac or skeletal. While cardiac cells usually provide stronger contractions and can contract autonomously, skeletal muscle tissue can be arranged in more complex three-dimensional shapes and the onset of contractions can be precisely controlled. Contractile cells isolated from insects, although they might be less researched or more difficult to manipulate, provide great advantages (Webster-Wood et al., 2017). Cells from invertebrate animals, in particular dorsal vessel (DV) tissues, are environmentally robust to extreme temperatures, pH conditions or pressures, metabolically adaptable and relatively autonomous (Baryshyan et al., 2012).

4.1 Insect-muscle-based biohybrid robots

Akiyama et al. fabricated a biohybrid robot from extracted DVs from the moth Thysanoplusia intermixta that was able to move due to friction differences between contractions and relaxation and achieved speeds of around 500 µm/s after the addition of a neuroactive chemical that increased its contraction frequency (Akiyama et al., 2012). Previously, the same group already demonstrated the feasibility of using cultures of insect cells without temperature control that resulted in spontaneously contracting tissue (Akiyama et al., 2008) and later presented a long-term and room temperature hybrid bioactuator from DV of the lepidoptera larva Ctenoplusia agnata, using micropillars that could measure their force and kept working at room temperature for more than 90 days without medium change (Akiyama et al., 2009). The use of chemicals for regulation of contractile response of DV tissue with several neuropeptides was also proved (Akiyama et al., 2008) and, recently, Tanaka et al. demonstrated the fabrication of a biohybrid pump powered by earthworm muscle, chemically modulated by the addition of acetylcholine, although with a slow response time of 42 s (Tanaka et al., 2019). Moreover, Webster et al. fabricated a 3D-printed biohybrid robot capable of crawling at speeds of around 80 µm/s (Webster et al., 2016). Isolation, optimization of culture conditions and cryopreservation of embryonic muscle cells from tobacco hornworm Manduca sexta larvae was studied by Baryshyah et al., demonstrating the expression of contractile proteins like myosin heavy chain and the presence of sarcomeric structures (Baryshyan et al., 2012). This study provided significant advances toward the development of established methods to culture, cryopreservation and differentiation embryonic myoblasts from insects, although there is still need a for scalability of the approaches to achieve extended use of DV tissue in biohybrid robotics. Nonetheless, later on, they demonstrated the fabrication of a 3D bioactuator that could self-repair, survive for months without medium changes and that was tolerant to different temperature and pH conditions, producing stresses of 2 kPa, which are comparable to those reported for skeletal muscle biorobots (Raman et al., 2016).

4.2 Cardiac-muscle-based biohybrid robots

Early approaches of cardiac-based biohybrid devices consisted on polymeric cantilevers that could be deflected by the contractions of seeded cardiomyocyte cells and could also be used to measure their forces (J. Park et al., 2005, 2006). Parallelly, Xi et al. and Kim et al. reported the fabrication of untethered biohybrid robots of three legs (Xi et al., 2005) and six legs (J. Kim et al., 2007), respectively, that could move due to friction differences. The three-legged biorobot could achieve speeds of 38 µm/s while the six-legged biorobot could move at estimated speeds of 87 µm/s. Likewise, Feinberg et al. fabricated and characterized muscular thin films made of PDMS and functionalized with fibronectin for cardiomyocyte attachment (Feinberg et al., 2007). These muscular thin films could adopt engineered 3D conformations like coils or helical shapes, and they demonstrated the creation of a walker and a swimmer with the same method (Figure 6a).

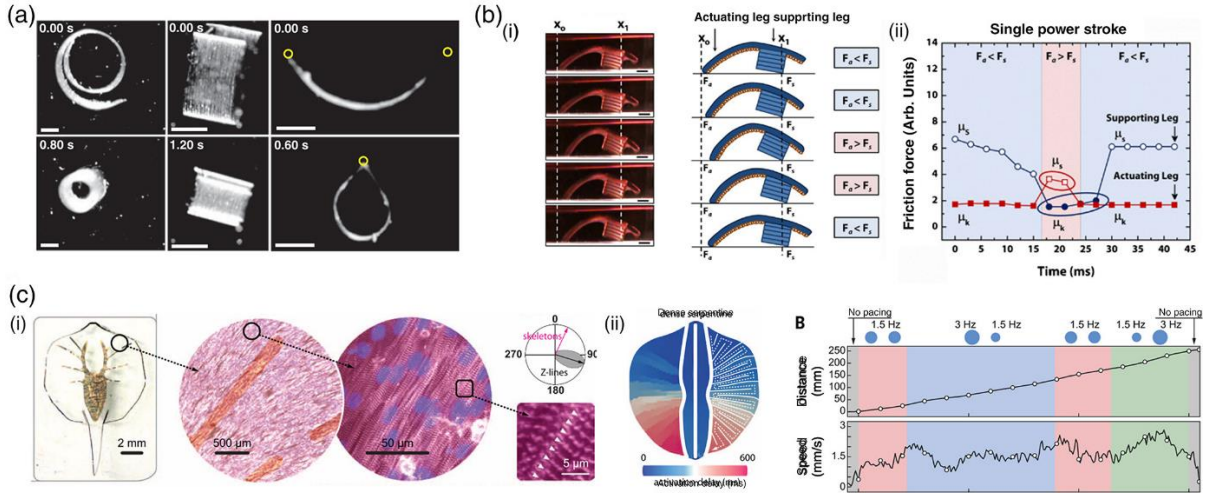

FIGURE 6
Biohybrid robots based on cardiac muscle tissue. (a) Snapshots of muscular thin films with different conformations upon contractions. Adapted with permission from Feinberg et al. (2007). (b) (i) Snapshots and motion mechanism due to friction of a biohybrid robot based on a thin film of cardiomyocytes. (ii) Plot of friction vs force of each one of the legs of the biobot (supporting and actuating), indicating that motion happens when the coefficient of static friction of the actuating leg is larger than the coefficient of kinetic friction of the supporting leg. Adapted with permission from Chan et al. (2012). (c) (i) Image of a biohybrid stingray made of a mold-casted PDMS structure stabilized with a gold skeleton and cardiomyocytes seeded on top. Right-hand images were immunostained for sarcomeric –actinin (red) and cell nuclei (blue), revealing its sarcomeric structures. (ii) A dense serpentine pattern of fibronectin was used to guide the attachment of cardiac cells and engineer a wave propagation pattern that simulated the deflection of a stingray's fins. (iii) Kinematic analysis of the distance traveled and speed of a biohybrid stingray upon different types of optical stimulation. Adapted with permission from S. J. Park et al. (2016)

In the following years, more complex and engineered biorobots based on cardiac muscle made appearance, although all of them built on the same principle of thin films. In 2012, Chan et al. developed a 3D-printed biobot, based on a poly(ethylene glycol) diacrylate (PEGDA) skeletons of different designs that could move at speeds of more than 200 µm/s (Figure 6b) (Chan et al., 2012). Their biobot was composed of a hard base and a softer cantilever, seeded with a cardiac cell sheet, that could deflect this thin film and push the biobot forward by creating differences in friction during a power stroke. Based on a similar design, Holley et al. fabricated cardiac-based swimming biorobots that could self-stabilize upon external disturbances (Holley et al., 2016).

Two of the most complex examples of biomimetic and biohybrid reverse engineering are the cases of the tissue-engineered medusoid and stingray (Nawroth et al., 2012; S. J. Park et al., 2016). To fabricate a reverse-engineered jellyfish, Nawroth et al. observed the propulsion and anatomy of jellyfishes and designed their own medusoid, using perfectly located and aligned cardiomyocytes on PDMS thin films that could replicate the stroke kinematics of a jellyfish (Nawroth et al., 2012). By analyzing their motion and dynamics of the surrounding flow fields, they found that their medusoid achieved speeds of around 0.5 body lengths per stroke, within the range of actuation of natural jellyfish. Later on, Park et al. recreated the body and motion of a stingray, using mold-casted PDMS structure and a microfabricated gold skeleton as flexible and rigid parts of the body, respectively, followed by a serpentine-patterned attachment of cardiomyocytes (Figure 6c). This serpentine pattern was a key element to achieve the undulatory motion of a stingray fins by delayed propagation of the contraction waves. Moreover, they genetically modified the cardiac cells to respond and contract to blue light on demand, achieving steering and speed control reaching almost 3 mm/s (S. J. Park et al., 2016).

Biohybrid systems actuated by cardiomyocytes have also been used to measure contractile forces, with possible applications in regenerative medicine and pharmacological testing (J. Kim et al., 2008; Knight et al., 2016; Legant et al., 2009; J. Park et al., 2005). Lind et al. fabricated a completely 3D-printed microphysiological device based on a thin film of seeded rat cardiomyocytes that could measure differences in stresses after the addition of drugs like the L-type calcium channel blocker verapamil and the β-adregenic agonist isoproterenol (Lind et al., 2017). Using multimaterial 3D printing, they printed on the same process: the base PDMS layer, strain gauge wires made of a thermoplastic polyurethane ink loaded with carbon black nanoparticles (CB:TPU), tissue guiding microfilaments, electrical and contact pads with a polyamide ink filled with Ag nanoparticles (Ag:PA) and finally, PDMS wells and insulation. The deflections of the cardiomyocyte-seeded thin film upon contractions could be measured thanks to the piezoresistive properties of the CB:TPU ink, obtaining direct measurements of the stresses generated.

4.3 Skeletal-muscle-based biohybrid robots

Skeletal muscle is currently the most used tissue for biohybrid engineering due to its controllability and adaptability (Ricotti et al., 2017). Although it is generally weaker than cardiac tissue, skeletal muscle poses several advantages that benefit its use. Unlike cardiomyocytes, skeletal muscle is inherently three-dimensional, making it easier to engineer in different structures, without relying in thin film architectures. Moreover, except during differentiation or myogenesis, spontaneous contractions are scarce and electrical, optical or neural stimulation are needed to activate the tissue, resulting in a higher level of on/off control. Biohybrid actuators that biomimic the muscle–tendon unit (MTU) of the musculoskeletal system of mammals have been fabricated as a possible alternative to hard or soft robotic grippers that do not possess the sensitivity and control of native muscle tissue (Figure 7) (Ricotti et al., 2017). Hereof, Morimoto et al. fabricated a biohybrid actuator based on an antagonistic pair of skeletal muscle tissues (Morimoto et al., 2018). Their device, which resembled a human finger with extended degrees of motion, could be bent in opposite directions by the selective alternative contraction of the adjacent muscles thanks to external gold electrodes located at a close distance (Figure 7a). The skeletal muscle tissues, extracted from neonatal rats, could achieve forces of tens of mN for up to 20 days and they proved the possibility of object manipulation using one or several of these biohybrid devices (Morimoto et al., 2018). Recently, the same group presented a similar biohybrid actuator that could be operated in air by being encapsulated in a collagen matrix with a perfusion system to maintain the necessary humidity conditions (Figure 7b) (Morimoto et al., 2020). Although the range of movement was decreased when compared to the previous version of the bioactuator, these results entail a step forward toward muscle-based bioactuators that could potentially replace actuators in soft robots, while enduring changes in environmental conditions.

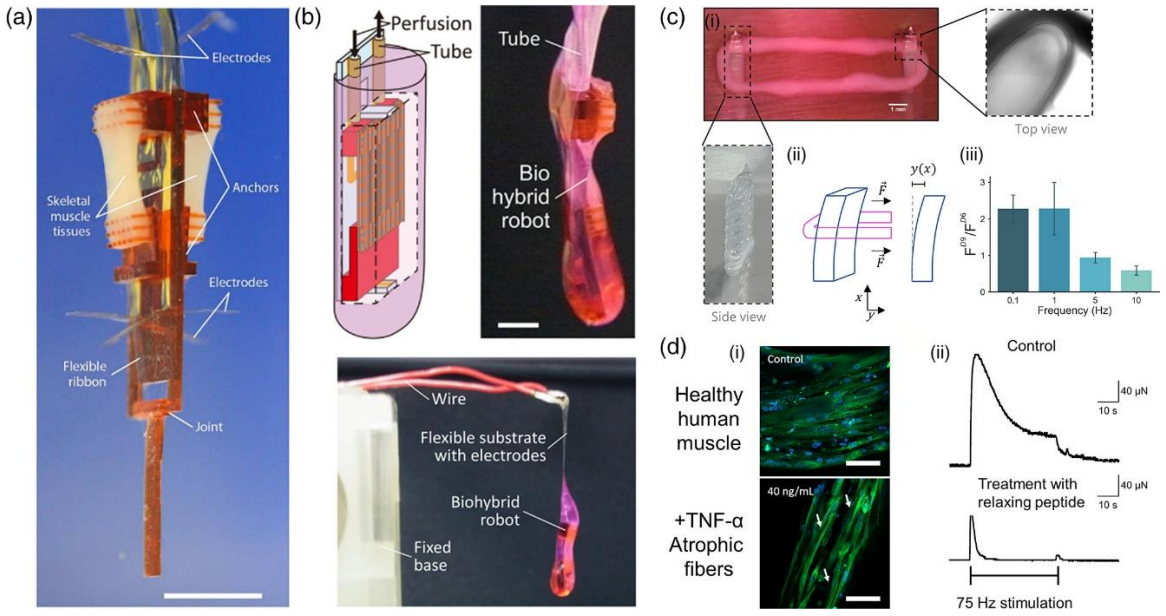

FIGURE 7
Biohybrid actuators based on skeletal muscle tissue. (a) Image of a biohybrid actuator based on an antagonistic pair of skeletal muscle tissues. Scale bar: 5 mm. Adapted with permission from Morimoto et al. (2018). (b) Schematic representation and real images of a biohybrid actuator covered with a collagen structure for actuation in air. Perfusion tubes allow the flow of culture media to the muscle tissue. Scale bar: 2 mm. Adapted with permission from Morimoto et al. (2020). (c) (i) completely 3D-bioprinted bioactuators composed of two PDMS posts surrounded by skeletal muscle tissue, (ii) which can be used as a force measurement platform. Iii) force increase after exercising the bioactuators with electric pulses of different frequency over a period of 4 days. Adapted with permission from Mestre, Patiño, Barceló, et al. (2019). (d) (i) Immunostaining images of young human skeletal muscle myocytes in the force measurement platform and aged-induced with atrophic fibers after the addition of 40 ng/mL of the cytokine TNF-α. scale bars = 80 μm. (ii) Contraction force profiles after a sustained stimulation of 75 Hz, showing the relaxing effects of a cosmetic peptide, proving the use of the bioactuators as a drug testing platform. Adapted with permission from Mestre, Garcia, et al. (2020)

Simpler bioactuators based on skeletal muscle tissue can be useful for fundamental research in tissue development, bioengineering of relevant tissue models to study diseases, regenerative medicine or drug screening. Most of these tethered bioactuators are composed of a set of soft cantilevers surrounded by a tissue ring that can bend them upon induced contractions, and can be used with both cardiac (Boudou et al., 2012; Legant et al., 2009) or skeletal muscle tissue (Sakar et al., 2012; Vandenburgh et al., 2008). Vandenburgh et al. used microposts to measure the force of murine microtissues upon the addition of several drugs, such as insulin-like growth factor 1 (IGF-1) and atorvastatin (Vandenburgh et al., 2008). Later, Sakar et al. employed optogenetically modified skeletal muscle cells to prove the local control of the contractions by applying concentrated light pulses, also with potential applications as a drug screening platform (Sakar et al., 2012). Recently, an improvement toward higher versatility and through-put was achieved by the fabrication of a completely 3D-bioprinted force measurement platform (Mestre et al., 2018; Mestre, Patiño, Guix, et al., 2019). Two PDMS posts and a myoblast-laden hydrogel were bioprinted in the same process, leading to a self-assembly of the tissue around the posts during differentiation and allowing to measure the forces exerted by the muscle upon electrical stimulation (Figure 7c). With this setup, the adaptability of skeletal muscle tissue after long-term exercising protocols was studied, focusing on the effects of stimulation frequency and stiffness of the post and finding force responses similar to the expected for native tissue.

However, for these biomedical applications, the use of human-derived myoblasts, instead of murine cells as in the previous cases, is preferred to obtain more relevant results before performing clinical trials. On this subject, some three-dimensional studies of tissue engineering of human cells have been performed, focusing on the effects of mechanical stimulation (Powell et al., 2002), cyclic preconditioning (Moon et al., 2008) or obtaining clinically relevant responses (Madden et al., 2015). In a recent study, the 3D-printed force measurement platform described in the previous paragraph was adapted for 3D bioengineered human muscle myoblasts (Mestre, Garcia, et al., 2020). Tumor necrosis factor α (TNF-α), a cytokine related to inflammatory processes and sarcopenia was used to induce a morphological and functional phenotype of aged or atrophic tissue, characterized by a decrease in myocyte diameter and deteriorated response upon electrical stimulation (Figure 7d). With this human muscle model, the relaxing effects of a cosmetic peptide were characterized, focusing on the kinematics of the contraction profiles at high frequency and demonstrating the capabilities of this platform to be used for drug screening purposes of human muscle in the biomedical or cosmetic industries.

Untethered biohybrid robots based on skeletal muscle tissue have also received attention in the past years. Cvetkovic et al. fabricated a biobot using 3D printing techniques and 3D bioengineering of muscle myoblasts. The biobot consisted of two T-shaped legs with a bridge structure joining both parts and could move with a crawling mechanism (Figure 8a). They optimized the stiffness of the material and analyzed the passive forces exerted by the tissue during myogenesis. Moreover, they compared the efficiency of symmetric and asymmetric biobots, both experimentally and through finite element analysis (FEA) simulations, proving that an asymmetry is needed to produce differential friction coefficients that allowed the biobot to move at speeds reaching more than 150 µm/s (Cvetkovic et al., 2014). Later on, Pagan-Díaz et al. studied the scalability of the same devices, fabricating stronger, larger and faster biological machines with different muscle configurations, supported by computational simulations based on a friction difference mechanism (Figure 8b) (Pagan-Diaz et al., 2018). Other long-sought aspects, such as self-healing, adaptability or integration with neurons or embedded electronics were later proved by the group using the same type of system (Cvetkovic et al., 2017; Y. Kim et al., 2020; Raman et al., 2016, 2017).

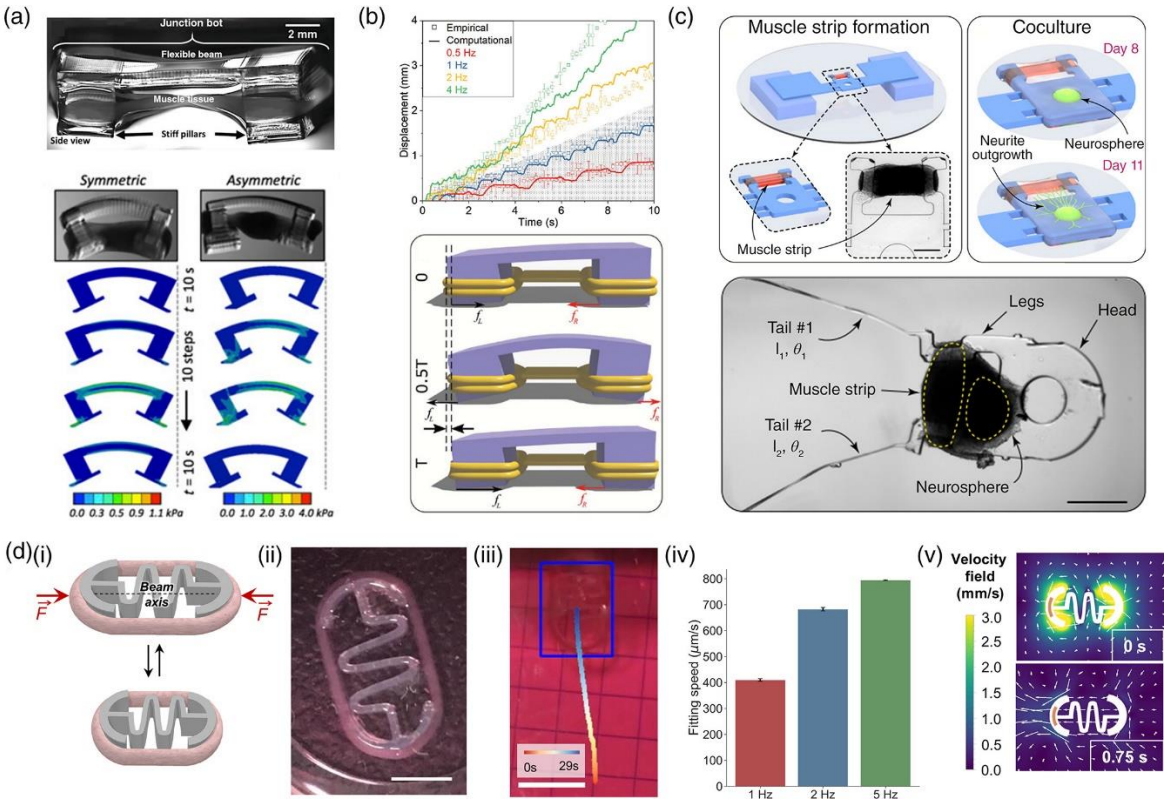

FIGURE 8

Biohybrid robots based on skeletal muscle tissue. (a) Image representing the formation of a biobot. On the bottom, comparison of the motion of a biobot with a symmetric skeleton and with an asymmetric one via finite element analysis (FEA) simulations, showing that only the asymmetric skeleton produces net motion. Adapted with permission from Cvetkovic et al. (2014) and Pagan-Diaz et al. (2018). (b) Displacement in time of large biobots at different frequencies, compared with numerical simulations of their motion. On the bottom, a schematic of the working mechanism by friction differences due to the asymmetry. Adapted with permission from Pagan-Diaz et al. (2018). (c) Schematic representation of the coculture of neurospheres with skeletal muscle tissue after 8 days of differentiation of the muscle, for the fabrication of a biohybrid swimmer. Normally, at day 11, neurite growth can be already observed. (d) Below, optical microscopy image of the biohybrid swimmer after release from its anchors, indicating the position of the muscle tissue and the neurospheres, which are activated via optical stimulation to induce contractions and swimming motion. Adapted with permission from Aydin et al. (2019). (d) (i) schematic representation of the spring-based muscle-powered swimmer and (ii) optical image of the assembly. (iii) Tracking of the biohybrid robot swimming at 1 Hz for 30 s at the air-liquid interface. (iv) Speed of the swimmer at different frequencies and (v) hydrodynamics FEA simulations of the flow around the skeleton during a contraction, showing a heterogeneous distribution of the flow lines due to the asymmetry in the design, which leads to motion. Adapted with permission from Guix et al. (2020)

4.4 Multicellular biohybrid robots

Most of the control mechanisms of hybrid biorobots have been based on electrical stimulation, mimicking the effect of neuronal action potentials, or optical stimulation, through the optogenetic modification of cells to depolarize their membranes upon the presence of light. Multicellular approaches offer the possibility of stimulation with neurons, although these approaches pose a significant challenge in the fabrication of more complex and controllable biohybrid robots. Webster et al. employed neuromuscular tissue directly isolated from the sea slug Aplysia californica to power an inchworm-inspired biohybrid actuator (Webster et al., 2017). The addition of carbamylcholine chloride, a drug that activates acetylcholine receptors, was used to induce neural stimulation, resulting in significantly larger muscle tension. Aydin et al. presented a biohybrid swimmer based on skeletal muscle tissue in coculture with neurospheres containing motor neurons (Aydin et al., 2019). The bioswimmer could form functional neuromuscular

junctions (NMJs) and optical stimulation of the neurospheres could, in turn, induce contractions in the muscle tissue (Figure 8c). Although the biohybrid swimmer did not attain very fast speeds compared to other examples in the literature due to the difficulty of motion at low Reynolds number, it represents the first biohybrid swimmer based on skeletal muscle in coculture with motor neurons, which could offer scalability to bioengineer larger and more efficient multicellular robots. Recently, Guix et al. presented a biohybrid swimmer based on a 3D-printed spring serpentine skeleton and skeletal muscle (Figure 8d) (Guix et al., 2020). The compliant nature of the spring skeleton provided an extra level of mechanical stimulation due to its restoring force when spontaneous contractions occurred during maturation, yielding larger force than tissues matured in static conditions. The biohybrid robot could swim in two different modes (at the air-liquid interface of liquid-bottom interface), depending on the configuration on the skeleton and could attain speeds of 800 µm/s, becoming the fastest skeletal-muscle-based swimmer up to date and with speeds comparable to its cardiac counterparts.

Other multicellular approaches have focused on engineering of tendon-like interfaces that better resemble the structure of native musculoskeletal tissue. Promising advances were carried out by Merceron et al., who 3D-bioprinted an MTU made of a stiff part containing fibroblasts and a softer part with skeletal myoblasts (Merceron et al., 2015). This MTU showed high viability, appropriate tensile properties and expression of relevant genes in the muscle–tendon junction. This 3D-bioprinting approach could be further integrated in previously reported biohybrid devices to improve the force transfer efficiency (Ricotti et al., 2017). Not only neurons and fibroblasts are relevant for the reproduction of native muscle. Endothelial cells, that form the vasculature, are also essential for the proper delivery of nutrients, especially in thick tissues. On this regard, 3D bioprinting has emerged as a viable tool for the biofabrication of complex multicellular tissue constructs and vascular networks based on sacrificial polymers that could give rise to the next generation of multicellular biohybrid robots (Datta et al., 2017; Hinton et al., 2015; Kamm et al., 2018; Kang et al., 2016; Kolesky et al., 2014, 2016; W. Wu et al., 2011).

Biohybrid robotics powered by muscle cells is a very heterogeneous field with a wide range of possibilities, especially when multicellular approaches are investigated. Several challenges, such as the need of vascularization, constant nutrient supply or controlled physiological conditions to ensure long-term stability makes difficult the creation of fully automated robotic systems. Therefore, most of the reported biohybrid devices are rarely taken a step forward, particularly toward biomedical applications such as drug testing or muscle tissue modeling, where they might find their most straightforward adoption before they can be applied in full robotic systems. The rise of more scalable techniques, such as 3D bioprinting, could be a solution to some of the challenges, like reproducibility or a smooth multimaterial integration, that might hinder their applications in biomedical research. Drug screening platforms, skeletal muscle or cardiac disease modeling could take advantage of a large part of the research carried out in biohybrid actuators and robots, but communication between very multidisciplinary fields like soft robotics, tissue engineering, biomedicine or nanotechnology will be necessary to obtain more meaningful research.

5. CONCLUSIONS

5.1 Challenges and outlook

Research on hybrid biorobotics is increasing at a high rate and will continue to do so in the following years and decades. On the one hand, while enzymatically propelled nanomotors are already starting in vivo trials with animal models, there is still a lack of understanding of their motion mechanisms or their interactions in the crowded environment of cells and tissues. More investigations into these fundamental aspects will be necessary to optimize the fabrication and performance of these devices, but also reduce their side effects. Future research should focus more on the fundamental interactions with biological matrices (Palagi et al., 2017; Walker et al., 2015; Z. Wu et al., 2018) and also in the interaction between several of these nanomotors, which would also offer an interesting system to study in active matter physics (Hortelao, Simó, et al., 2020; Illien et al., 2017). The effects elements like ionic species or pH on the motion efficiency still need to be further investigated, as seemingly contradictory results arise (Arqué et al., 2020; De Corato et al., 2020; Tang et al., 2020), hinting at a much more complex interaction between all the actors involved in the motion. Moreover, in practical applications, the formation of protein coronae (Feiner-Gracia et al., 2017; Nel et al., 2009) or interactions with the immunitary system (I. C. Yasa et al., 2020) or the in vivo imaging (van Moolenbroek et al., 2020) are some of the main challenges that need to be addressed to ensure their efficacy as drug delivery systems. Effective ways of protecting these nanomotors against their environment, new functionalities (e.g., targeting moieties, biosensing capabilities), imaging techniques (e.g., through PET-CT) or smart delivery approaches of encapsulated drugs could significantly increase the complexity and performance of nanomotors and micromotors. The next generation of hybrid smart nanomotors will undoubtedly include a wide library of elements or moieties that will increase their stability, efficiency, targeting and range of applications.

Biohybrid systems powered by single cells, especially those driven by bacteria, can show potential applications in the biomedical field as active drug delivery systems, for biofilm removal or in fundamental studies of motion at the microscale. However, for biomedical applications, there are some critical aspects that could make their widespread adoption difficult, namely the response of the immune system, their removal after administration, or the potential loss of engineered behavior (Hosseinidoust et al., 2016). Using the own person's bacteriome or genetically modifying common bacteria to reduce their toxicity have been proposed as possible approaches to circumvent these issues, but still the efficacy and safety of each system might need to be considered on a one-by-one case (Hosseinidoust et al., 2016).

Finally, biohybrid robotics based on muscle tissue are already reaching the limit of what can be achieved by simple integration of skeletal or cardiac muscle tissue with soft skeletons, with bioswimmers reaching speeds of the order of 1 mm/s. Further research should focus on the integration of several tissues and obtaining more complex, yet useful, ways of actuation that can finally prove the benefits of using native muscle tissue instead of man-made soft actuators. The integration of muscle with neural cells forming NMJs has already been reported in several publications (Aydin et al., 2019; Cvetkovic et al., 2017; Kaufman et al., 2020; Webster et al., 2017), although there are important challenges to be overcome regarding the coculture conditions, the fabrication methods or the scalability of the approaches. Other types of integration could include satellite cells to be able to induce self-healing when sarcomeres break after exercise (Orfanos et al., 2016), fibroblasts to better emulate the environment of the muscle–tendon unit (Merceron et

al., 2015) or vasculature to ensure proper delivery of nutrients (Kolesky et al., 2016). With more faithful models of 3D muscle tissue, tethered biological actuators could be excellent candidates for drug screening of muscular disorders that could use the patient's own cells or be used for muscle regeneration. The future generation of biohybrid robots will undoubtedly need to solve important long-term challenges regarding their stability, lifetime, assembly, or scalability. These main challenges reside in ensuring the long-term stability of the constructs, their tolerance to different environments, which might require the fabrication of novel and soft bioreactors to protect the tissue (Morimoto et al., 2020), and the improvement of the control mechanisms. The fabrication methods should evolve in such a way that integration with soft or flexible electronics or actuators can be feasible and scalable, while not affecting the performance or viability of the biological tissue. The addition of nanoparticles or smart responsive materials could add further functionalities to biohybrid robotic devices, in the form of energy conversion mechanisms (e.g., piezoelectric, magnetoresistive, plasmonics, and similar), control of actuation or material reinforcement. All these advances will certainly need to go hand by hand between 3D bioengineering and material science research, and communication between both disciplines will be essential.

5.2 Ethical considerations

The future perspectives of research in biohybrid robotics would not be complete without an acknowledgment of the ethical considerations that come into play when dealing with bioengineering or synthetic biology, which have not been extensively covered due to the novelty of the field (Raman & Bashir, 2017). Top-down approaches based on full tissue explants to power biorobots face several ethical concerns related to proper animal treatment at scientific facilities and overall ethical procurement of cells or biomolecules from them (Ricotti et al., 2017; Webster-Wood et al., 2017). Moreover, nanomotors and micromotors or cell-driven microswimmers, if used as drug delivery systems or similar biomedical applications, should be held to the highest health and safety standards even if their biocompatibility issues are improved with the use biocompatible enzymatic reactions or genetically modified bacteria. For this reason, their safety and toxicity should be thoroughly addressed in 2D cultures or 3D organoids before reaching clinical trials or even animal trials (Bredenoord et al., 2017; Otto et al., 2016). On this note, 3D bioengineering of tissues becomes a convenient approach to reduce the amount of animal trials in biomedicine or pharmacology research. The use of human-derived cells might also improve the relevance of this type of research by using tissue models that resemble human native tissue the most. However, this bottom-up approach carries more ethical considerations than top-down approaches, related to the protection of dignity and anonymity of patients, the use of embryonic cells or tissue ownership, among others (Bredenoord et al., 2017; Otto et al., 2016; Skloot, 2010). Moreover, although the use of a patient's own cells for personalized medicine could be revolutionary and improve the health of millions of people worldwide, there are several risks regarding social aspects of the use of this research, like their associated cost or their use as human enhancement technologies, which might not be accessible to everyone, increasing the breach of social inequality (Kamm et al., 2018; Otto et al., 2016). On the other hand, one of the most crucial questions resides in whether these fields could be considered to be creating life, replicating it or reusing it for other purposes (Kamm et al., 2018). This type of language raises fundamental arguments of religious, cultural and political nature that are currently difficult to be settled. The development of biohybrid robotic systems will entail, undoubtedly, an increase in debates of an ethical nature in the near future, especially when more complex devices comprising neural tissue with certain intelligence start to be developed. Sensitivity to all cultural perspectives and potential

risks is paramount for ethical research, acknowledging the possibility of precautionary approaches that require stepping down until clear and updated governmental policies are defined to protect society from unethical or corrupt exploitation of this research. The scientific community should not adopt an observing and passive role, but acknowledge and assess both precuationary and proactionary approaches (Kamm et al., 2018), together with a conscious communication with bioethicists, transparent transmission of research discoveries and participation in risk assessment of the possible dangers.

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AUTHOR CONTRIBUTIONS

Rafael Mestre: Conceptualization; writing-original draft; writing-review and editing. Samuel Sanchez: Conceptualization; resources; writing-original draft; writing-review and editing. Tania Patiño: Conceptualization; writing-original draft; writing-review and editing.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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