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Stimuli-Responsive Engineered Living Materials

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Review

Stimuli-Responsive Engineered Living Materials

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Stimuli-responsive materials are able to undergo controllable changes in materials properties in response to external cues. Increasing efforts have been directed towards building materials that mimic the responsive nature of biological systems. Nevertheless, limitations remain surrounding the way these synthetic materials interact and respond to their environment. In particular, it is difficult to synthesize synthetic materials that respond with specificity to poorly differentiated (bio)chemical and weak physical stimuli. The emerging area of engineered living materials (ELMs) includes composites that combine living cells and synthetic materials. ELMs have yielded promising advances in the creation of stimuli-responsive materials that respond with diverse outputs in response to a broad array of biochemical and physical stimuli. This review describes advances made in the genetic engineering of the living component and the processing-property relationships of stimuli-responsive ELMs. Finally, the implementation of stimuli-responsive ELMs as environmental remediators, biosensors, drug delivery vehicles, and soft robots is discussed.

1. Introduction

Stimuli-responsive materials sense and respond to environmental conditions and enable devices with programmed functionalities, designed for applications in biomedicine,[1,2] wearables,[3,4] sensors,[5,6] actuators,[7–9] electronics,[10] and soft robotics.[11] Typically, the external cues that induce material changes include pH, light, temperature, chemicals, humidity, or electrical fields. In response, these materials can be designed to morph in shape,[12–14] change color,[15] heal,[16] degrade,[17,18] and perform other functions.[19] Stimulus-response in many materials is typically triggered by relatively strong stimuli. Here, we define strong stimuli as changes in environmental conditions that would be likely to cause undesired changes to other materials or living organisms surrounding the stimuli-responsive material. In many proposed applications of stimuli-responsive materials, including a variety of medical devices, the need for a strong stimulus is often a limiting factor. One approach to creating polymers that respond with high specificity to weak stimuli, such as biochemical changes, is to carefully synthesize designed binding motifs on the polymer.[20,21] While some biomolecules might be relatively easily detected, such as enzymes, many less reactive molecules are difficult to detect. Stimuli-responsive materials that respond with high sensitivity has attracted the attention of material scientists to create materials that mimic the functions and behaviors of living organisms. Although significant efforts have been developed,[22,23] it is still challenging to create materials that perform complex biological functions such as chemotaxis, adaptation, growth, and metabolic functions in response to highly specific and weak stimuli. Living organisms respond to weak stimuli in ways that are encoded by the information in the genome. This response can be inherent to the organism or programmed to enhance sensitivity to surrounding the environment. To survive, cells must process dynamic changes in the form of mechanical and biochemical signals that are poorly differentiated (e.g. diastereomers). Living cells are capable of adapting to their environment, are highly efficient metabolic machines, and are often genetically manipulable.

Engineered living materials (ELMs) integrate living and non-living components. They can harness the biological potential of cells to enable dynamic, self-assembling, and functional materials. [24–27] Previous reviews that describe ELMs focus on engineering biological cells that act as living factories or modulate the performance of novel materials,[25] programming cells to produce materials with functional properties, [26] and integrating cells with synthetic materials to develop sensors and actuators.[27] For example, ELMs have been developed to utilize engineered cells for the synthesis of materials, such as amyloid proteins that form biofilms,[28–30] cellulose,[31] and other polysaccharides.[32] These extracellular materials have been investigated to perform different functionalities, such as self-regeneration and adhesion to surfaces. For the purpose of this review, we focus on describing stimuli-responsive ELMs, where living cells are incorporated within materials and are used to endow materials with stimuli-responsive functions. These materials offer opportunities to program specific responses in devices that require weak and poorly differentiated stimuli to perform diverse functions.

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To fabricate stimuli-responsive ELMs, the design of a well-engineered matrix to address control, stability, and survival of the living component is necessary. Several strategies for cell immobilization in or on polymer materials have been implemented, such as adsorption on surfaces, emulsification, extrusion, coacervation, and spray drying.[33] Synthetic hydrogels are often used as scaffolds and embedding matrices because they provide a protective environment for living cells and allow the exchange of nutrients and waste.[34–36] Strategies used to create ELMs are related to the strategies of tissue engineering, where macroscopic tissues and organs are created by controlling mammalian cell proliferation and differentiation.[37,38] Nevertheless, mammalian cells are fragile. Small changes in their environment can result in death and their growth conditions require well-controlled maintenance for long-term survival. By comparison, bacteria, yeast, and microalgae are substantially more robust. Microorganisms thrive in a wide range of environmental conditions because of their adaptive and metabolic behavior. These living cells act as sensing machines that detect small, weak molecule concentrations or changes in natural or physiological environments. Importantly, advances in genetic engineering technologies enable a wide range of possibilities to design living cells with programmable sensing and functions. Characteristics such as high sensitivity and specificity to weak stimuli, robustness, orthogonality, continuous sensing, and scalability can be achieved.[39,40] In order to build stimuli-responsive ELMs, cells with such characteristics must modulate the physical or chemical properties of the material or modify the surrounding environment upon exposure to external stimuli.

In this review, we briefly present relevant advances in the field of synthetic biology for the design of stimuli-responsive ELMs that sense the external environment and respond in a controlled manner. Next, we explore strategies for the manufacturing and processing of these materials (Fig. 1). Finally, we highlight different applications of stimuli-responsive ELMs in environmental and biomedical fields.

2. Engineered living materials design and processing

The design of materials that actively respond to the environment and perform programmed functionalities can be achieved by incorporating genetically engineered living microorganisms. Living cells such as bacteria, yeast, and microalgae can be tailored to sense and detect changes in their environmental conditions. Encapsulating these cells in materials enables the fabrication of ELMs with stimuli-responsiveness. Genetically engineered cells can be incorporated to engender a range of inputs, such as single chemicals or light, and programmed outputs, such as the production of enzymes or the expression of proteins. Reporters have been in use in molecular biology for decades. Their application to ELMs enables precise quantification of cell proliferation, fluorescence, bioluminescence, or colorimetric parameters within materials.[41] The union of genetic manipulation and materials science enables the fabrication of stimuli-responsive ELMs for a broad range of practical applications.

2.1 Synthetic biology for the development of stimuli-responsive ELMs

Synthetic biology enables the reprogramming of the biological functions of microorganisms to achieve desired and specific responses.[42–45] Engineered prokaryotic and eukaryotic cells can be used as stimuli-responsive elements for ELM devices, where generally weak signals detected by the cells can produce output

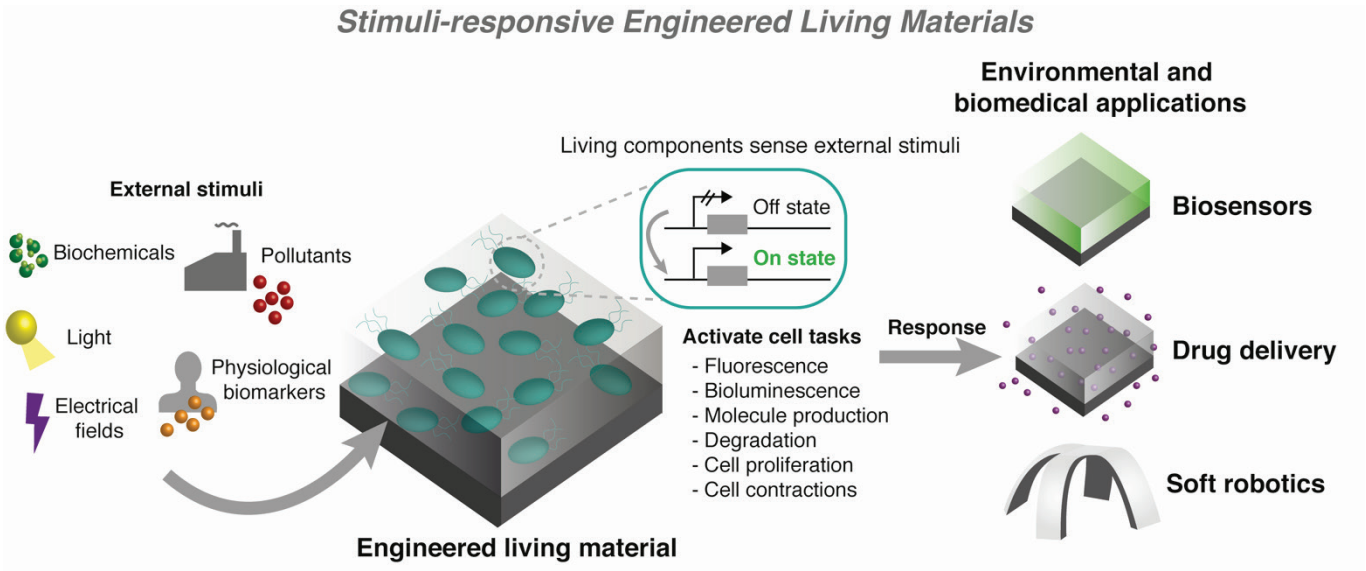

Fig. 1 Stimuli-responsive engineered living materials. Schematic representation of an ELM that sense the external environment and respond in a controlled manner. Materials that contain genetically engineered living components are capable of sensing specific stimuli. Upon detection, cells can mediate a broad range of ELM responses. These include practical environmental and biomedical applications, such as biosensing, drug delivery, and robotic function.

signals represented by a change in cell proliferation, function, or metabolic activity. These changes can directly modulate the properties or functions of the device, for example, by changing the material's shape to create soft actuators or by releasing molecules locally for drug delivery. One common route to create an ELM is to modify the embedded living components to express reporter genes that encode for proteins that produce measurable signals in response to target stimuli.[46] Common reporters used produce a fluorescent, colorimetric, or bioluminescent signal when a chemical or physical stimulus is detected (e.g., biochemicals, light). Fluorescent reporters such as green fluorescent protein (GFP), from the jellyfish *Aequorea Victoria,* can be employed to identify cells, protein localization, and transcriptional activity. GFP can be readily visualized, is non-toxic for cells, and does not require the presence of substrates other than oxygen for the maturation of the chromophore.[47] Enzymatic reporters such as β-galactosidase, from *Escherichia coli* (*E. coli*) encoded by the lacZ gene, react with an external substrate and yield a product that can be detected by colorimetric assays.[48] Bioluminescent systems have evolved independently on well over 30 occasions.[49] In eukaryotic applications, the sea pansy (*Renilla*), copepod (*Guassia*), and firefly enzymes are the most common. In bacterial reporters, the *Vibrio* luciferase alpha and beta chains are used to emit light.[50,51] The use of reporters permeates the life science as they have been used extensively to monitor protein-protein interactions, transcription, recombination events, and transduction efficiencies.[47,52,53] Notably, when implementing these reporter systems within materials, ELMs can be leveraged to create biosensing devices that detect specific signals and report by changing the physical properties of the material (e.g., production of visual outputs). The fabrication of stimuli-responsive ELMs offers exciting opportunities in the development of environmental remediation and biomedical technologies.

Gene expression can be activated or repressed.[54] For example, transcriptional induction can occur in response to chemicals such as isopropyl β-D-1-thiogalactopyranoside (IPTG).[55] IPTG causes a transcriptional repressor to be inactivated and enables production of genes under the control of a lac operator sequence. There are a range of promoters that can be utilized to enable a range of chemical stimuli. By coupling sensing elements to reporter genes, cells can report the presence of a specific chemical inducer and initiate production of reporter genes such as GFP (Fig. 2A).[56,57] Control of gene expression by chemical induction is slow, concentration dependent, and can require generations to reverse. Applications when dynamic control of cell behavior is necessary require more precise controls.[58] Optogenetic switches make use of light to control gene expression. They can provide fine spatial and temporal control of gene expression. Optogenetic switches have been introduced in bacteria, yeast, and mammalian cells for drug screening, cell signaling, biosynthesis of target molecules, and control of mechanical responses.[58–62] For example, genetically engineered *E. coli* were constructed with an optogenetic switch that activated intracellular drug production upon illumination with blue light.[63] Thermal bioswitches have been constructed with expression systems that activate at different transition temperatures.[64] For example, *E. coli* was modified to express GFP after external induction with focused ultrasound or after detecting fever within a mammalian host.[64] Other genetic circuits have been engineered to sense changes in pH for the dynamic regulation of extracellular organic compounds.[65] For example, when using sugar acids, such as D-xylose, as substrates for cell growth, the substrate oxidates to D-xylonic acid and acidifies the media. This media acidification can be detrimental to cell growth. To address this issue, a genetic circuit that utilized a pH-responsive receptor protein was tested in *E. coli* to control D-xylonic acid accumulation by detecting changes in extracellular pH.[65] Other sensing circuits engineered in living cells have been used for the detection of heavy metals,[66,67] organic compounds,[68] and biomarkers.[69] The responses obtained from these engineered living cells can enable new levels of control over the functions of synthetic materials.

The development of engineered cells for environmental and biomedical applications has rapidly improved. Methods to optimize genetic circuit performance have focused on lowering limits of detection, increasing selectivity, and modulating dynamic range.[70] Signal processing methods include the integration of logic gates to detect multiple target stimuli or genetic amplifiers to enhance expression and increase dynamic range to ensure good signal-to-noise ratios.[70–72] Logic gates allow the programmed recognition of multiple inputs to trigger a desired output.[73,74] For example, genetic circuits that respond to both pH and temperature have been engineered to perform complex logical AND or NAND operations involved in the regulation of GFP expression.[75] Programmed bacterial strains have been integrated in 3D structures to perform complex logic functions by interacting with each other and with external chemical inducers (Fig. 2B).[76] Methods that involve genetic amplifiers have been studied to increase sensitivity and improve transcriptional input signals with large output dynamic ranges. These signal amplifiers have been proposed to be used in environmental applications where pollutants could be detected at low concentrations.[77] For example, an arsenic responsive circuit, built with a fixed-gain amplifier, was engineered in *E. coli* to generate a GFP output signal in response to arsenite concentrations as low as 0.25 μM. Genetically programmed cells that detect the variety of stimuli described above and perform intricate signal processing tasks will open new opportunities in the design of stimuli-responsive ELMs.

Manufacturing methods to introduce engineered cells in appropriate matrices are needed to design stimuli-responsive ELMs. In the next section, we will describe fabrication methods that incorporate living organisms into devices for use in environmental remediation, biosensors, drug delivery, soft robotics, self-healing, and self-cleaning applications.

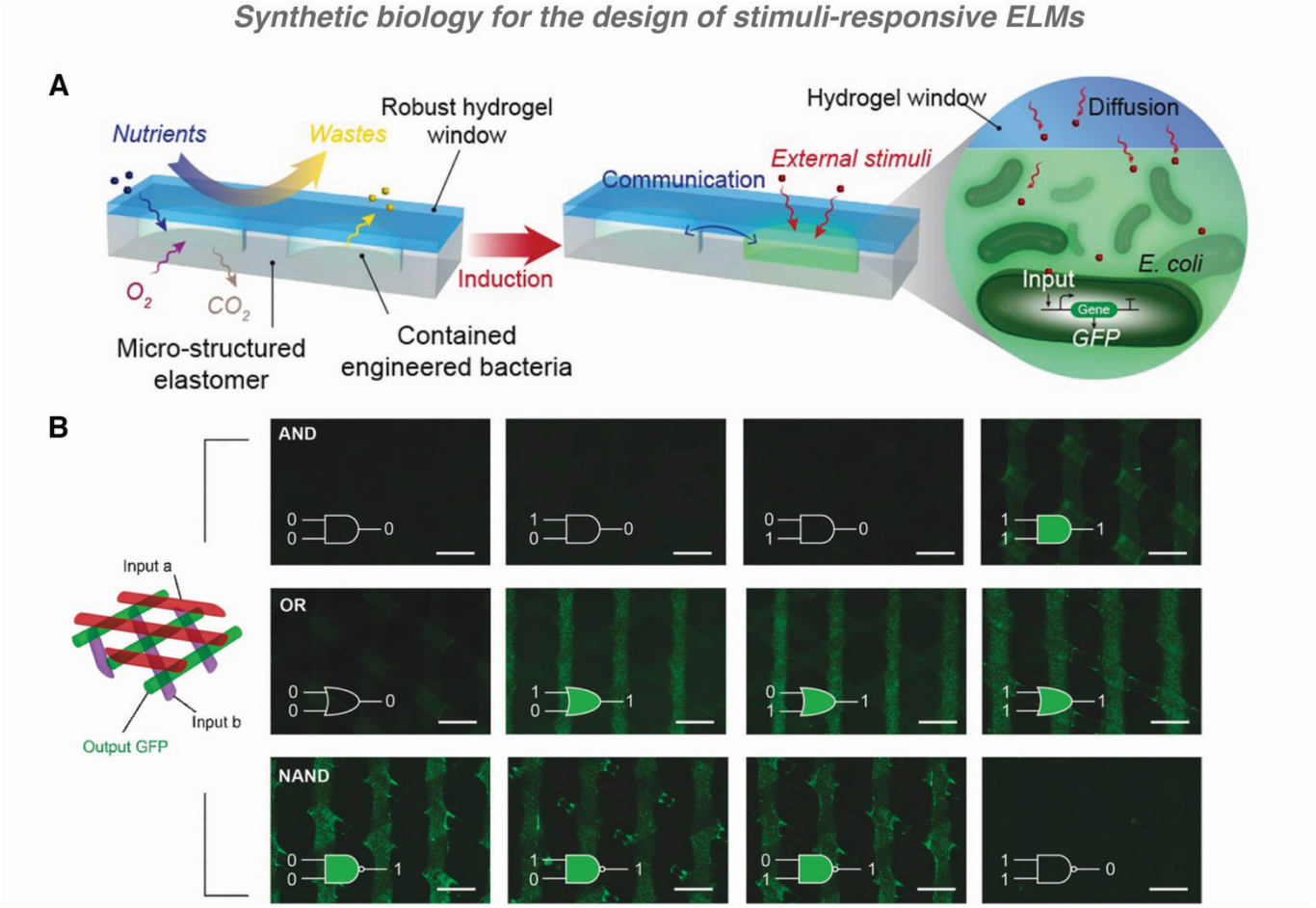

Fig. 2 Synthetic biology for the design of stimuli-responsive ELM devices. (A) Schematic representation of an ELM with stimuli-responsive properties. The material incorporates living bacteria genetically engineered to express a fluorescent protein (GFP) in response to external stimuli. The living component allows the ELM to produce an optical signal that can be detected and measured. Reproduced with permission from ref. (56). Copyright (2017) PNAS. (B) Logic gates achieved in 3D-printed, stimuli-responsive ELMs. Programmed bacterial strains embedded in 3D printed structures perform computational operations (AND, OR, NAND). These cells act as outputs that detect the presence or absence of chemical components within the same structure and report by the presence or absence of GFP production after achieving a specific logical function. Adapted with permission from ref. (76). Copyright (2017) Wiley-VCH.

2.2 Processing techniques for the manufacturing of stimuli-responsive ELMs

Processing techniques can be implemented to generate stimuli-responsive ELM devices. Typically, living cells are encapsulated or immobilized in soft matrices by chemical or physical crosslinking, adsorbed on surfaces or membranes, or encapsulated using microfluidic techniques.[78–80] In each of these approaches, traditional materials processing concerns, such as geometric control, microstructural control, and throughput, are combined with a need to maintain the viability of the living cells. We will highlight both 3D-printing and electrospinning as powerful approaches to create ELMs with programmable properties. 3D-printing enables constructs with site-specific control over cell distribution in a pre-designed format. Electrospinning of polymer-cell composites enables the fabrication of fabrics with controllable architecture and mechanics. These processing techniques enable nano, micro, and macroscale control to construct 3D environments suitable for cell encapsulation. As many processing methods to build complex ELM structures described in the literature encompass these techniques, we emphasize relevant work that uses such technologies. Finally, we will also describe additional processing methods for the production of ELMs.

The fabrication of ELMs with 3D printing and electrospinning techniques enables digitally-defined structures. Most frequently, 3D-printed ELMs are printed using extrusion-based techniques, such as direct-ink-write printing. Direct-ink-write printing makes use of biologically active microorganisms contained in a soft pre-gel matrix with shear-thinning properties, typically called “bioink.” Materials with these properties can be extruded under shear forces and have the ability to maintain the shape of the sheared structure before further crosslinking. Within these bioinks, the unpolymerized matrix should be compatible with the cells and serve as a protective matrix against stresses induced during the printing or electrospinning process and should maintain cellular functions after crosslinking. Hydrogels are typically used in the synthesis of bioinks because they are able to mimic an environment that maintains the biological functions of the living component. Common examples of biocompatible hydrogels include the use of natural and synthetic networks such as agarose, alginate,

gelatin, collagen, fibrin, polyacrylamide, polyethylene glycol diacrylate, among others.[81–84] Natural hydrogels offer high biocompatibility and cell viability but typically consist of physically crosslinked networks with weak mechanical properties. Synthetic hydrogels with chemically crosslinked networks permit cellular function and tunability of the mechanical properties. The development of bioinks with enhanced printability and biocompatibility have been explored by introducing functional groups via crosslinking or by using reinforcing materials that can enhance printing and electrospinning resolution.[85] Bioinks with improved mechanical integrity and print fidelity, while maintaining biocompatibility and cellular functions, continue to progress.[86–89]

Strategies that utilize 3D-printing technologies to develop structures with control over cell distribution and cell density have been reported. For example, multiple bacterial species 3D-printed to form living microstructures have been created to study cell-cell interactions and behaviors.[90] In another study, bacterial spores were printed within agarose gels to create living materials capable of surviving extreme conditions and detecting chemicals or harmful bacteria when germinated.[91] Moreover, digital fabrication platforms have been described to control chemical distribution within 3D printed biohybrid objects and facilitate interactions between genetic constructs and chemical signaling profiles.[92] Other examples used mixtures of alginate and *E. coli* transformed to express red fluorescent proteins. These mixtures were printed on the millimeter-scale to create physically crosslinked structures that responded to external chemical inducers.[93] The development of functional living inks to 3D print multiple bacterial strains has been demonstrated in the design of living materials with pre-determined functionalities for bioremediation, adaptive behavior, and biomedical applications (Fig. 3A).[94,95] Besides the advances in bacterial processing, other microorganisms such as yeast and microalgae have been utilized to manufacture ELMs. For example, *Saccharomyces cerevisiae* has been printed within high-resolution scaffolds at low and high cell concentrations for the production of ethanol from glucose fermentation.[88,89] Microalgae were deposited in a layer-by-layer manner to create algae hybrids and study microalgal cell behavior and long-term viability.[96] Microorganisms from multiple kingdoms, like bacteria, algae, and yeast, were distributed within the same 3D-printed structure to study viability and cellular growth behaviors (Fig. 3B).[97] This approach could offer new understandings in the way different species behave within the same encapsulating structure or provide methods to create ELMs for cell-cell communication and interactions with the external environment or the encapsulating matrix.

Electrospinning is a highly versatile processing method that utilizes electrostatic forces to assemble micro/nanometer-scale non-woven polymeric fibers with high porosity and large surface area. It has been implemented in the encapsulation of microorganisms for delivery systems of probiotics, molecule sensing, agriculture, wastewater bioremediation, and drug delivery (Fig. 3C, D).[98–100] Probiotic *Lactobacillus* species are viable in encapsulated polyethylene oxide nanofibers after electrospinning.[101] In the encapsulation of fungi, *Kluyveromyces lactis* and *S. cerevisiae* were combined with polyvinyl alcohol or cellulose acetate to produce electrospun ELM nanofibers. These structures can remove aflatoxin B2, which is a toxic metabolite with adverse effects produced by fungi found growing on agricultural products.[102] In general, 3D-printing and electrospinning approaches allow the top-down fabrication of structures that contain living organisms. One major advantage of these additive processing strategies is that both processes facilitate the fabrication of ELMs with multiple species. The co-location of multiple species is a physical strategy that can be used to complement genetic engineering strategies to enable greater functionality in response to a single stimulus or the ability to respond to multiple stimuli. These approaches mirror work where disparate synthetic materials are built into responsive structures; for example, structures that respond to both temperature and pH can be fabricated.[103] Incorporating multiple species in one structure could enable high specificity and selectivity to a variety of external stimuli, for example, by reporting the presence of multiple biomarkers in the same environment.

Additional techniques used for the fabrication of ELMs include wet spinning and roll-to-roll processes.[104,105] Both wet-spinning and roll-to-roll techniques are highly scalable. For wet spinning methods used in ELMs, polyvinyl alcohol (PVA) microfibers containing dispersed *Micrococcus luteus* or *Nitrobacter winogradskyi* bacteria were used for gold sequestration and nitrate bioremediation, respectively. Using energy-dispersive X-ray spectroscopy and transmission electron microscopy, it was shown that the embedded *M. luteus* had successfully sequestered the gold into the composites. For bioremediation, the fibers encapsulating *N. winogradskyi* were capable of oxidizing nitrite and it was demonstrated by observing a decrease of nitrite concentration surrounding the fiber.[104] A roll-to-roll continuous coating process was demonstrated to build an ELM based on engineered *Bacillus subtilis* endospores. The cells were encapsulated in a PVA hydrogel and were cast onto a nonwoven poly(ethylene) terephthalate support. It was observed that cells retained functionality during this process as they were capable of generating a fluorescence signal after IPTG sensing.[105]

In summary, the combination of synthetic biology and materials science offers new methods to develop stimuli-responsive ELMs with well-defined functions. High specificity to a variety of molecules can be programmed into cells to produce composites that sense, respond, and modify the physical or chemical properties of the material itself or the surrounding environment. By implementing processing tools to control the spatial distribution of cells, living responsive devices for different applications can be designed.

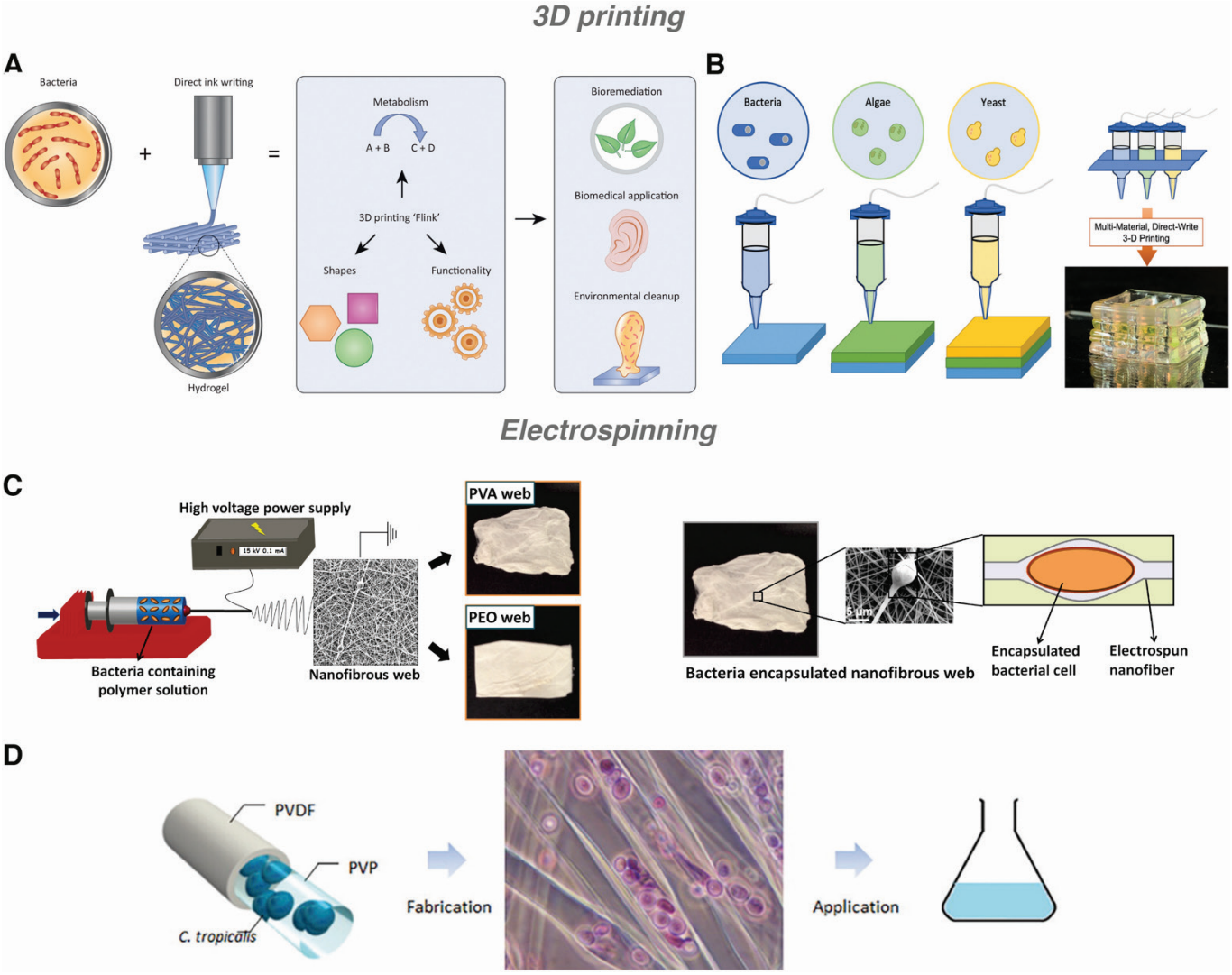

Fig 3. Fabrication strategies for the development of stimuli-responsive ELMs. (A) Schematic representation of a 3D-printing process for the fabrication of functional printable living materials that can be implemented in biomedical and environmental remediation applications. Reproduced with permission from ref. (95). Copyright (2018) Elsevier Ltd. (B) 3D-printing approaches can be used to build structures that contain multiple cell types. Adapted with permission from ref. (97). Copyright (2020) Wiley-VCH. (C) Schematic of an electrospinning process for the encapsulation of bacteria within nanofibrous webs. Picture and SEM micrograph of bacteria encapsulated in electrospun fibers. Reproduced with permission from ref. (99). Copyright (2017) Elsevier Ltd. (D) Yeast cells were encapsulated in core-shell polymeric nanofiber. Micrographs showing yeast cells encapsulated within the fibers. Reproduced with permission from ref. (100). Copyright (2015) American Chemical Society.

3. Applications of stimuli-responsive engineered living materials

The applications described in this section focus on ELMs with stimuli-responsiveness for environmental and biomedical challenges. The combination of synthetic biology tools to address the control of living cells and processing techniques allows the fabrication of ELMs to build devices for sensing in environmental and biomedical applications, devices for drug delivery platforms, and soft biohybrid robots.

3.1 Environmental monitoring

Wastewater effluents rich in toxic heavy metals, organic compounds, and other pollutants are released into soil and water by modern industrial processes.[106] Several processes have been used to remove these contaminants, such as the use of harsh chemicals for heavy metal precipitation, ion-exchange, flocculation, reverse osmosis, evaporation, and ultrafiltration. However, these methods are not able to effectively mitigate pollution completely and require very costly measures that generally result in the production of secondary toxic by-products and residues.[107,108] In this context, bioremediation, which capitalizes on naturally occurring components found in bacteria, fungi, and plants, is harnessed to transform wastes into less harmful products. In so doing, the goal is to restore the habitability and natural resources of a given ecosystem. Many organisms are capable of detecting contaminants and using them as an energy source. Enzymes can be produced that stimulate the breakdown of pollutants through biochemical modifications.[109,110] Sensing and monitoring techniques with the use of living cells were first described in 1975 using the bacterial strain *Acetobacter xylinum*, for electrochemical

detection of ethanol.[111] This work is among the first examples of cell-based sensors, in particular for fermentation and environmental applications. In 1988, baker's yeast cells were incorporated into carbon paste matrices for the fabrication of a high-sensitive bioelectrode for the detection of primary alcohols.[112] With the advances in synthetic biology, genetic engineering of cells allowed the development of new technologies for the detection of carcinogens[113] and harmful chemicals,[114] or the removal of toxic compounds.[115] For example, seminal work that focused on the development of genetically engineered bacterial *Pseudomonas* strains, encoding genes for the degradation of petroleum hydrocarbons such as camphor, octane, xylene, naphthalene, and salicylate, opened new pathways for the microbial biodegradation of organic pollutants.[116–119] Since then, technologies for the removal of pollutants have focused on the design and evolution of microbial and cell plant-assisted bioremediation pathways.[107,116,120]

Materials that encapsulate microorganisms for bioremediation have demonstrated promising results in the sensing, monitoring, and treatment of environmental pollutants.[121,122] ELMs offer unique advantages over synthetic stimuli-responsive materials and traditional sensors for pollutant monitoring. Stimuli-responsive materials in the form of filtration membranes and adsorbents are capable of modulating membrane pore size, permeability, and wettability. However, the stimuli used to induce these changes is often strong, including highly acidic or basic solutions, and need to be carefully controlled to selectively separate pollutants from contaminated wastewaters.[123–128] Paper-based materials that detect the presence of pollutants with high selectivity have been designed to produce a fluorescent readable signal in response to specific heavy metals and nitroaromatic pollutants.[129–131] However, the probes that are attached to these materials for detection could slowly diffuse out when exposed to solvents and, therefore, reduce sensor accuracy and sensitivity. Strategies that use semi-interpenetrating hydrogels, coordination polymers, or metal-organic frameworks have been used to detect metals and organic compounds; however, they often require organic solvents to function, are moisture sensitive, and suffer from low selectivity, which can lower their use in practical applications.[132–135] Traditional biosensors and detection technologies are often limited by detection capabilities and often fail to provide relevant information about pollutant bioavailability.[52,136] Cell-based biosensors have proven useful in bioavailability assessment and monitoring of pollutants.[137] However, despite the advances in cell-based sensing technologies, there are still very few commercially available sensors due to biosafety concerns over the stability and the release of genetically engineered microbes to the environment.[70] Strategies that prevent these organisms from escaping experimental environments are highly desired for reducing potential risks associated with damages to the ecosystem.[138,139] Long-term viability is necessary to ensure functionality, reproducibility, and stability during long-term storage. Immobilization techniques can offer long-term detection or remediation activity and allow the reuse and recovery of the living cells after pollution treatments. Stimuli-responsive ELM sensors show promise for environmental applications because of the renewability, high scalability, and inexpensive cultivation of cells, as compared to conventional sensors and stimuli-responsive materials. High sensitivity in stimuli-responsive ELM sensors can be achieved to detect specific pollutants at weak concentrations. This can be obtained with the use of genetic engineering and signal processing techniques, in combination with high-throughput reporting methods such as fluorescence and bioluminescence expression. New routes for the use of this class of materials in sustainable technologies for efficient selection, detection, and degradation of uncontrolled pollutants in the environment, such as metals and organic compounds, are described in this section.

3.1.1 Monitoring systems for the detection of contaminants

Stimuli-responsive ELMs offer opportunities for the efficient detection of pollutants in various environmental sources. Engineered cells that respond to such contaminants have been integrated within synthetic materials to sense target molecules with high specificity and sensitivity. Because the sensing mechanism involved in these materials is governed by the living cells, the stimuli used to induce a response is specifically targeted to the cells and is usually weak. Metals such as copper, often found in contaminated drinking water and soil, at high concentrations can potentially cause health effects in humans and harm aquatic ecosystems. Towards the design of a metal-responsive ELM, metal-sensing *E. coli,* engineered to express GFP as a function of metal ion concentration, was used as a sensing agent with high selectivity toward copper ions. Cells were encapsulated within polyacrylamide hydrogels to create sensors that undergo a fluorescence quenching effect in response to concentrations of copper ions. These sensors were capable of efficiently detecting copper (Cu^{2+}) at concentrations down to 5 mM and displayed a linear relationship with the level of fluorescence quenching.[140] Traditional stimuli-responsive materials that change their mechanical properties in response to copper ions have been studied to switch between hard and soft states.[141] Comparing the level of sensitivity between copper-responsive ELMs and copper-responsive polymers, we observe that ELMs respond to lower concentrations of copper (5 mM Cu^{2+}) as compared to the concentrations needed to generate a desired response in copper-responsive polymers (0.1 – 1.0 M Cu^{2+}).[141] This suggests that ELMs remain functional even when subjected to weak stimuli. Organic compounds, such as estrogens, pose a serious risk to the environment.[142] A study that developed a bacterial cellulose-based ELM system was used to sense and respond to environmental concentrations of the estrogen β-estradiol (BED).[143] This report utilized *S. cerevisiae* engineered to express a BED-activated transcription factor (Z_3EV) and a GFP reporter under control of the Z_3EV promoter. Bacterial cellulose produced from the bacterium *Komagataeibacter rhaeticus* was used as a cellulosic matrix to incorporate the responsive yeast. The resulting ELM was used to sense the presence of the chemical inducer BED at

concentrations of 5 nM and, in response, it was capable of producing a strong GFP signal throughout the material.[143] Hydrocarbons pose potential risks to human health and the environment.[144] Stimuli-responsive ELMs for the detection of hydrocarbons have typically used genetically-engineered bioluminescent bacteria. These bioreporters, developed by DNA recombination, integrate genetic constructs consisting of the *luxCDABE* gene, derived from the marine bacteria *Vibrio fischeri*.[50] For detection of toxic chemicals, the bioreporter is capable of emitting light by starting the transcription of the *lux* gene cassette to produce luciferase. This synthetic biology strategy has been implemented to detect naphthalene and salicylate, using the bacterial bioreporter *Pseudomonas fluorescens* HK44.[145] This engineered strain harbors the pUTK21 plasmid, which carries *nah* genes that encode for the degradation pathway of naphthalene, linked to the *lux* gene cassette coding for bioluminescence. A study that used silica films with immobilized *P. fluorescens* HK44 was developed to test the effects of naphthalene and salicylate on bioluminescence of the sensor. Bioluminescence from the sensor was detected after 50 min of induction with the hydrocarbons and reached its maximum after 4.5 h. Minimal detection limits to induce bioluminescence were found to be 1.2 mg/L for naphthalene and 0.5 mg/L for salicylate. Selectivity was tested using 32 different possible inducers, which only 3 were detected to induce a bioluminescent signal similar to the naphthalene and salicylate signals.[145] A similar method integrated a bioluminescent reporter, to build a toluene-responsive ELM. *Pseudomonas putida* TVA8 was engineered with a *tod-luxCDABE* bioreporter that responds to the presence of toluene, benzene, ethylbenzene, and xylene. Cells were encapsulated in a silica matrix attached to the end of a quartz optical fiber. The ELM was exposed to toluene concentrations between 5.3 mg/L and 26.5 mg/L, and bioluminescence was detected after 12 h of exposure regardless of the concentration.[146] These strategies enable materials with bioluminescent behaviors that can be readily measured by photon counting detection systems. A major strength of bioluminescent outputs is the remarkable signal to noise ratios resulting from the use of photons of visabel light as an endpoint.

Taking advantage of encapsulation methods to create a device with multiple responses to various toxic compounds, a dip-stick type biosensor that produces bioluminescence in response to a pool of contaminants has been developed.[147] The dip-stick biosensor consisted of eight bioluminescent bacterial strains encapsulated in color-coded alginate microbeads that were entrapped in a laser cut transparent glass (Fig. 4A). These bacterial strains were constructed with stress promoters that start the transcription of the *luxCDABE* gene to produce luciferase when the cells detect different toxic chemicals. Specific modes of stress responses such as DNA, oxidative, membrane, and protein damage were triggered in the cells by five model chemicals, *i.e.*, Mitomycin C (MMC), 1-methyl-1-nitroso-*N*-methylguanidine (MNNG), paraquat, hydrogen peroxide (H_2O_2), and 2,4-dicholorophenol (2,4-DCP). Each microbead showed their own stress response to the chemicals by producing a bioluminescent signal when the device was exposed to contaminated water sources (Fig. 4B).[147] This approach shows a significant advancement for the development of stimuli-responsive ELM devices that detect

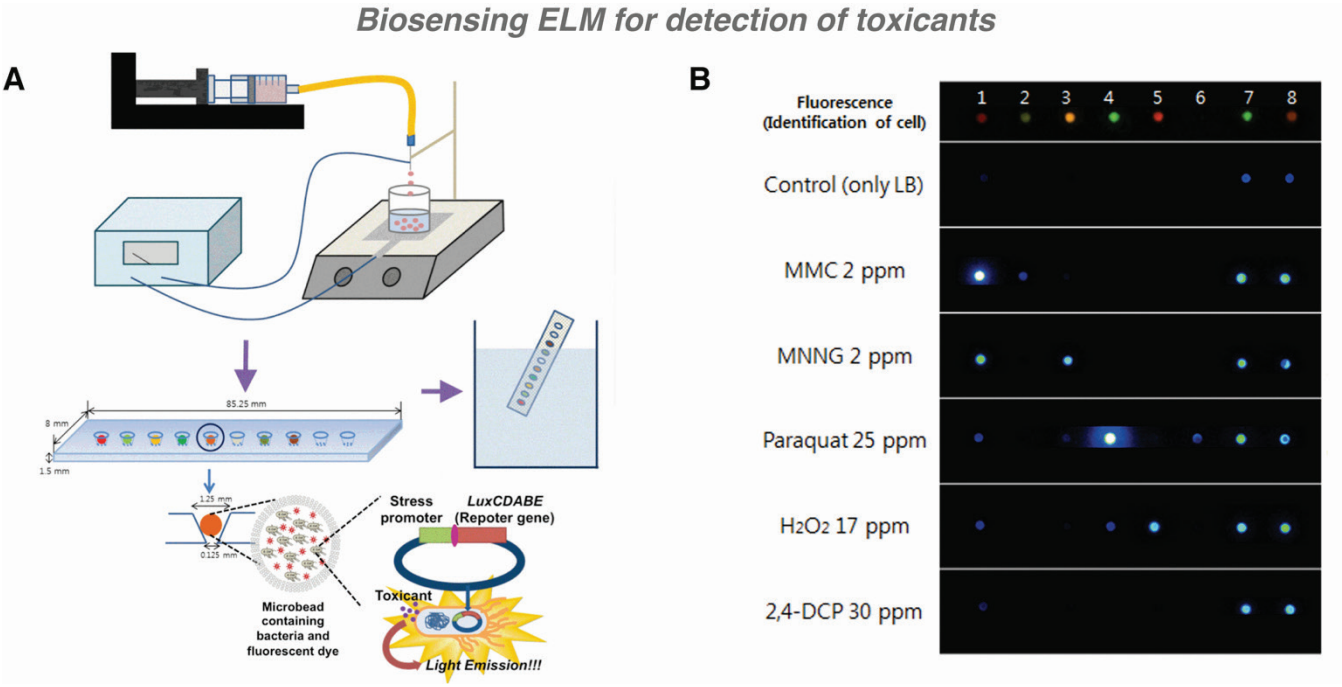

Fig 4. Dip-stick type biosensor for the detection of toxicants in water. (A) Schematic representation shows the process of building a dip-stick biosensor that contains genetically engineered bioluminescent bacteria for the detection of multiple pollutants. (B) Dip-stick biosensor bioluminescent response to five different model chemicals. Adapted with permission from ref. (147). Copyright (2014) Royal Society of Chemistry.

multiple chemical contaminants with specificity and produce a readable and easy to measure response. An overview of the applications described in this section is given in Table 1.

3.1.2 Ongoing challenges and future directions

Stimuli-responsive ELMs involved in the detection of contaminants in the environment use cells as bioreceptors to detect the presence of specific target pollutants. The key advantage of designing these materials is that synthetic biology enables the engineering of cells with incredible capabilities to respond to low amounts of target pollutants. This highly sensitive and specific detection can be measured through quantitative analysis of output gene expression. Processing techniques enable ELMs with multiple responses to several input signals, which increase their applicability in the field. Nevertheless, cell encapsulation remains a key challenge. Carriers need to be carefully selected when building stimuli-responsive ELMs. Most materials used for encapsulation consist of soft matrices, such as hydrogels, because they provide an environment that retains and exchanges nutrients for long term viability, increase metabolic activity, and provides protection.[148] Encapsulation allows high efficiency of cell immobilization within the material, which facilitates and improves testing readouts from output signals. However, sensitivity of encapsulated cells is often lower than that of their non-encapsulated counterparts because of the limited diffusion of nutrients, oxygen, and target molecules through the polymeric matrices. When employing ELMs for extended periods of time, the encapsulating matrix stability needs to be considered. Another key challenge is cell leakage from the ELM. Engineered cells that escape from the device could outcompete natural organisms and adversely affect the environment. Uncontrollable release of cells from ELMs also contributes to low signal-to-noise ratios, thus decreasing biosensing performance. Encapsulation techniques described in this section make use of silica matrices. These matrices are usually porous, provide the efficient diffusion of target molecules for monitoring, and prevent cell leakage, but long-term viability can be limited by these stiff materials. We expect that the design of future stimuli-responsive ELMs for environmental applications could have a focus on decontamination of harsh, toxic pollutants from the environment.

3.2 Biomedical applications

3.2.1 Biosensing technologies for molecule detection and diagnostics

The integration of genetically engineered cells into materials enables stimuli-responsive ELM wearables for healthcare monitoring. Non-living, sensing wearables have been extensively studied to monitor temperature,[149] strain,[150] pressure,[151] and metabolites found in body fluids[152,153] with selectivity and high sensitivity.[154,155] They offer significant advantages for non-invasive and continuous real-time monitoring for diagnostics, especially when the wearables are designed to collect data wirelessly. Nevertheless, incorporation of electrodes, flexible printed circuits, and power supply units are still needed to design high-performance, functional devices. Stimuli-responsive ELMs to build wearables that do not require the use of power for data collection and processing, show significant promise in the screening of weak stimuli, such as target biomarkers, for diagnostics. For example, materials integrating bacteria programmed with genetic circuits have

Table 1 Stimuli-responsive engineered living materials for biosensors in environmental applications

Biological component	Synthetic material	Function	Stimulus[a]	Response	Ref.
Escherichia coli	Polyacrylamide or silica	Detection of copper ions	Copper ions (5 mM - 5M)	Fluorescence quenching	[140]
Saccharomyces cerevisiae	Bacterial cellulose	Detection of estrogen	β-estradiol (5 nM)	Fluorescence expression	[143]
Pseudomonas fluorescens	Silica	Detection of hydrocarbons	Naphthalene (1.2 mg/L) and salicylate (0.5 mg/L)	Bioluminescence	[145]
Pseudomonas putida	Silica	Detection of toluene	Toluene (5.3 mg/L - 26.5 mg/L)	Bioluminescence	[146]
Eight different *Escherichia coli* strains	Alginate	Detection of multiple toxic compounds	Mitomycin C (2 ppm), 1-methyl-1-nitroso-*N*-methylguanidine (2 ppm), paraquat (25 ppm), hydrogen peroxide (17 ppm), and 2,4-dicholorophenol (30 ppm)	Bioluminescence	[147]

been fabricated as living wearable sensing patches that adhere to the skin and wearable gloves with chemical detectors at the fingertips.[56] These devices consisted of tough, stretchable polyacrylamide-alginate hydrogel and silicone elastomer covalently bonded to create bilayers encapsulating different genetically engineered *E. coli* strains. These strains express GFP when detecting chemical stimuli, such as N-acyl homoserine lactone (AHL), IPTG, and rhamnose (Rham). Interactions between these chemicals and the living material are created via chemical diffusion through the hydrogel, where expression of GFP is observed as an output reporting signal when each bacterial strain detects their specific cognate inducer. As a result, the ELM sensing patches and gloves became fluorescent in their presence.[56] The same research group developed a 3D-printed living, stretchable tattoo that integrates these multiple GFP-expressing bacterial strains for the detection of chemicals applied on human skin. 3D-printable bioinks were synthesized that contained the programmed *E. coli* strains, Pluronic F127 diacrylate micelles to provide rheological behavior, photoinitiator to allow further crosslinking, and nutrient media to maintain cell viability. Upon exposure to each chemical inducer on the skin, a specific region of the tattoo produced fluorescence (Fig. 5A). The wearable device was capable of resisting skin deformation without showing any signs of detachment or damage.[76] Responsive ELMs for wearable textiles have utilized genetically engineered *E. coli* patterned on cotton or plastics, by inducing cell adhesion in response to colored light. An *E. coli* strain was designed to encode a *csg*BAC operon, controlled by a blue light promoter, involved in the synthesis of curli fibers that anchor the formation of biofilms. Additionally, it included another promoter involved in the production of GFP under green light. To create wearable ELMs, the engineered bacteria were first adhered on cotton fabric by forming a biofilm in response to blue light. Then, ELM response was assessed by incubating the living fabric and shinning green light at 532 nm to induce expression of GFP. GFP fluorescence was then visualized under blue-light transillumination. Living fabrics exposed to green light showed 65% more fluorescence than fabrics not induced by green light.[156] ELMs with a hygroscopic response to sweat have been used to build smart garments.[157] The wearable was designed by incorporating moisture-responsive *E. coli* onto moisture-inert films, like latex, to create ventilating flaps in a running suit. The responsive flaps in the suit opened when detecting body sweat from a wearer during exercise. The suit could control body temperature and could effectively remove body sweat (Fig. 5B, C). Additionally, the same study designed a fluorescent running shoe prototype that incorporated GFP-expressing *E. coli* on the ventilating flaps. The shoe's flaps bent and exhibited increased fluorescence intensities when humidity conditions increased around the sole of the shoe.[157] In general, even though these ELM wearables utilize engineered strains for proof of concept monitoring of chemicals and physical stimuli, devices that detect physiologically relevant molecules could open future work in the fabrication of stimuli-responsive ELM biosensors for real-time point-of-care diagnostics.

Materials used to sense biomolecules for the development of medical technologies are highly desired for their use in diagnostic applications and early detection of diseases. One approach is to use stimuli-responsive polymers for sensing weak stimuli, by including recognition units such as phenylboronic acid-based polymers that detect sugars and respond with a change in diffraction,[158] wettability,[159] or fluorescence quenching.[160] These materials are stable in physiological conditions, but frequently have poor selectivity for saccharides. Other approaches utilize enzyme-functionalized materials that contain moieties for the catalysis of specific reactions which, in turn, leads to physicochemical changes in the material. Such methods include color-changing hydrogels that respond to analytes, including urea and glucose.[161,162] Often, these enzyme-functionalized hydrogels suffer from poor stability because of their sensitivity to environmental conditions.[163,164] Stability and selectivity can be achieved with the use of molecularly imprinted polymers for the specific detection of biomarkers related to different diseases, like phenylketonuria, cancer and immune-suppressant disorders.[165,166] Often times, these polymers have poor water compatibility that can affect sensing performance and affinity.[167] Stimuli-responsive ELMs offer unique advantages in next generation biosensing technologies for detection and diagnostics.

Detection of physiologically relevant molecules, that are usually weak in nature, with stimuli-responsive ELMs has been achieved with biological paper analytical devices (bioPADs),[168,169] tough hydrogel biocontainment platforms,[170] and a wireless analytical device.[171] bioPADs composed of filter paper, ink, genetically engineered yeast, and hydrogel have been designed to detect doxycycline at concentrations down to 0.3 μg/mL. The living yeast biosensors utilized *S. cerevisiae* strains transformed with tetracycline responsive plasmids, linked to the red fluorescent reporter protein yEmRFP. The plasmids contain a reverse tetracycline transactivator, rtTA, capable of activating in the presence of the antibiotic doxycycline. The detection of the antibiotic promotes the transcription of the reporter gene to generate a readable fluorescent signal. The bioPAD was designed using paper filter printed with patterns that served to spot a pre-gel solution of sodium alginate containing the yeast. The gel was then crosslinked by submerging in calcium chloride to entrap the cells in the paper device. Results showed that bioPADs were capable of producing fluorescent signals when exposed to physiological fluids of human urine or bovine serum spiked with doxycycline (Fig. 5D, E).[169] *In vitro* sensing of physiological molecules can also be performed using tough polyacrylamide/alginate hydrogel biocontainment beads. These beads encapsulate engineered probiotic *E. coli* efficient in the detection of heme. Heme sensing is possible because cells carry an outer membrane hemin receptor (chuA) and a bioluminescent reporter. When heme is internalized through this transporter, it interacts with the transcriptional repressor HtrR, to then generate a bioluminescent signal from expression of the bacterial *luxCDABE* operon. The tough biocontainment

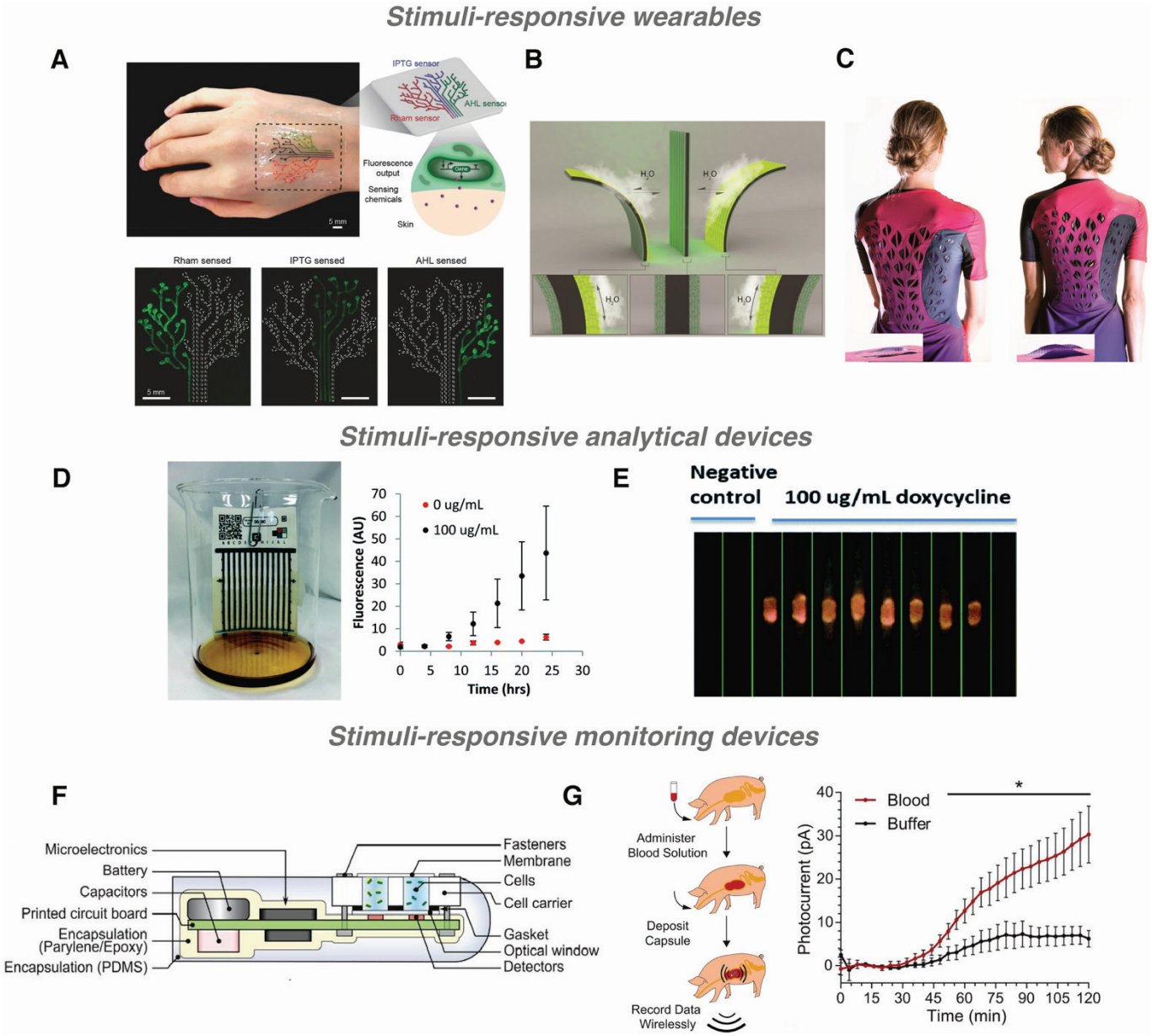

Fig 5. Stimuli-responsive ELMs for wearable, analytical and monitoring devices. (A) 3D-printed ELM tattoo encapsulating engineered bacteria that sense different chemicals on the skin. The tattoo is capable of reporting the presence of chemicals by emitting fluorescence. Adapted with permission from ref. (76). Copyright (2017) Wiley-VCH. (B) Moisture-responsive films that change shape under humidity conditions (C) Smart wearable suit with flat ventilating flaps before exercise (left) and bent flaps after exercise. Adapted with permission from ref. (157). Copyright (2017) exclusive liscensee American Association for the Advancement of Science. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC) http://creativecommons.org/licenses/by-nc/4.0/. (D) Fluorescent bioPAD spotted with modified yeast embedded in a hydrogel matrix. The device was developed in a reservoir of media containing doxycycline (left). Fluorescence was monitored over time in bioPADs treated with or without 100 µg/mL of doxycycline (right). (E) Device treated with 100 µg/mL of doxycycline was imaged on a fluorescence lightbox. Adapted with permission from ref. (169). Copyright (2020) Royal Society of Chemistry. (F) Electrical system diagram of the ingestible, monitoring device. (G) Schematic of a porcine model of gastrointestinal bleeding. Blood was administered, capsule was then deposited, and collected data was wirelessly transmitted (left). Photocurrent measurements over time of the device in the presence or absence of blood. Data was collected from the porcine model of gastric bleeding (right). Adapted with permission from ref. (171). Copyright (2018) American Association for the Advancement of Science.

ELMs were capable of sensing heme released from defibrinated blood and producing a significant increase in bioluminescence activity.[170] A monitoring device suitable for *in vivo* sensing of gastrointestinal molecules has been previously demonstrated by incorporating the above-mentioned heme responsive bacterial strain in a wireless readout capsule. The ingestible micro-bio-electronic device (IMBED) combines bacteria with microelectronics to enable local sensing of disease-related biomolecules (heme, AHL, thiosulfate) associated with the state of disease in the gastrointestinal tract. IMBEDs were built to detect bioluminescence generated by the engineered bacteria, using phototransistors that convert the signal to a digital code and transmit it wirelessly to an external device (Fig. 5F). The capsule was deposited into the stomach of a porcine model of gastrointestinal bleeding, and detection in response to heme was achieved in less than an hour after deployment (Fig. 5G). Small amounts of the molecule were able to be detected with high specificity and sensitivity.[171]

3.2.1.1 Ongoing challenges and future directions

Stimuli-responsive ELMs for wearable, analytical, and monitoring biosensors offer great opportunities in detection and monitoring of physiologically relevant molecules. The key advantages of using ELMs to detect molecules related to disease states include that the biosensors are highly sensitive and specific and can report through the activation of gene expression. However, their implementation in real-world biomedical applications is a key challenge. When using ELM wearables, living cells entrapped in fabrics or hydrogels are prone to dehydration and may have limited nutrient exchange with the human body. One potential opportunity to overcome this limitation is to engineered cells to survive for long periods of starvation or utilize available molecules, such as those found in sweat, as a form of energy source.[172] Future studies could have a focus on developing wearables that use engineered cells for targeted drug delivery, monitoring disease-related biomarkers, and measuring body temperatures on the skin. Analytical ELMs based on paper scaffolds provide control of nutrient and oxygen diffusion for efficient cell viability. They are generally suitable for high-throughput analysis of body fluids and are compatible with cells.[173] Wearables and analytical paper-based devices provide information from biomarkers found in blood, saliva, sweat, tears, and urine. However, one of the most important advantages of utilizing stimuli-responsive ELMs, is their versatility to be used for *in vivo* monitoring. For example, the gastrointestinal tract provides a rich source of biomarkers related to health and disease state. Genetically engineered cells have been studied to sense levels of gut biomarkers, report disease, and deliver therapeutics.[174] Nevertheless, a key challenge is that many biomarkers of interest cannot be detected because their receptors have not been fully studied or have not yet been identified.[175] This issue limits biosensor applicability. Some strategies described in this section use ELMs to monitor biomarkers related to gut disease. However, when translating these strategies for use *in vivo*, different aspects, such as the highly acidic environment of the stomach, areas of near anaerobic conditions, and highly variable transit time need to be considered to ensure sensor performance.[176] In addition, when building ELMs, a major design consideration is the selection of strains that colonize or do not colonize the gut, if cell leakage from the device is present. Some strains colonize the gut without disrupting the microbiome, and others could generate serious side effects.[177] We expect that future advances in synthetic biology greatly expand the use of stimuli-responsive ELMs that could be effective in the detection and quantification of complex, highly specific molecules for healthcare applications.

The design of ELM biosensors with stimuli-responsiveness to physiologically relevant biomolecules is of great interest in creating wearable or monitoring devices. Synthetic biology tools could enable the development of microorganisms that respond to multiple stimuli and could open new ways to facilitate the design of ELMs for personalized medicine. The use of microorganisms can be further explored to design ELMs for delivery of therapeutics. In the next section, we expand on the use of stimuli-responsive ELM devices that sense the environment and produce molecules for the design of drug delivery platforms. An overview of the applications described in this section is given in Table 2.

3.2.2 Drug delivery platforms

Integrating biological and synthetic components is a promising strategy to obtain specific control of drug delivery. Stimuli-responsive polymeric materials, such as responsive drug-loaded microcarriers, are often functionalized to recognize biological changes in the body that alter material properties such as solubility, shape, or state of aggregation.[178,179] Materials that perform these functions are desired to enable on-demand delivery of therapeutics, with spatial and temporal controlled release triggered by strong physical, chemical, or biological stimuli.[180] Different stimuli such as temperature, magnetic fields, ultrasound, light, pH, redox gradients, and enzymes have been investigated for the potential release of drugs at specific areas within the body.[181–186] These stimuli are often strong and non-specific and when used in physiological conditions, they can be triggered at undesired sites or they can disrupt the environment surrounding the material. Materials that carry a payload and detect these stimuli can change shape, burst, degrade, or solubilize. However, the local changes associated with disease are often subtle, which complicates the design of synthetic materials that can sense and respond to this disease state by releasing a drug. New design concepts that use metabolically-engineered bacteria or fungi have been proposed as they avoid previous drug manufacturing, drug encapsulation, and drug stability.[187,188] Synthetic biological therapies that use engineered bacteria have been reported for cancer therapy and diagnosis,[189–192] treatment for genetic conditions,[193] therapies for infectious conditions,[194] and treatment for gastrointestinal disorders.[195] However, some issues with colonization of these microorganisms could generate undesirable immune responses or infections. Microorganisms could freely circulate within the body or deliver a drug in a non-specific location, making these approaches complicated for clinical translations.[187] ELMs with stimuli-responsiveness to biological molecules or specific cues, may offer opportunities where living cells can be contained and used to deliver drugs at specific locations within the body. Importantly, the use of ELMs may provide ways to remove microorganisms when their functions are complete.

Towards the development of drug delivery ELMs, encapsulation of bacteria has been reported to endow materials with drug-producing capabilities. A study that utilized a bacterial strain of *Serratia marscescens* developed a stimuli-responsive ELM for the production of prodigiosin.[196] This red-pigmented metabolite, produced by the bacteria, has been found to have antimicrobial and anticancer activities.[197] Silica matrices were used to encapsulate the bacterial cells and nutrients were provided as external stimuli to induce the production of prodigiosin. Production of this metabolite was observed within

Table 2 Stimuli-responsive engineered living materials for biosensors in biomedical applications

Biological component	Synthetic material	Function	Stimulus	Response[a]	Ref.
Escherichia coli	Polyacrylamide/alginate and silicone	Wearables for the detection of chemical inducers	N-acyl homoserine lactone (100 nM), isopropyl β-D-1-thiogalactopyranoside (1 mM), rhamnose (12 mM), diacetylphloroglucinol (100 µM), and anhydrotetracycline (200 ng/L)	Fluorescence expression (GFP)	[56]
Escherichia coli	Pluronic F127 diacrylate micelles	Wearables for the detection of chemical inducers	N-acyl homoserine lactone (100 nM), isopropyl β-D-1-thiogalactopyranoside (1 mM), rhamnose (12 mM), and anhydrotetracycline (200 ng/L)	Fluorescence expression (GFP)	[76]
Escherichia coli	Cotton fabric	Wearables for the detection of light	Blue light (445 nm and 1 mW/cm²), green light (532 nm and 0.6 mW/cm²)	Fluorescence expression (GFP)	[156]
Escherichia coli	Latex	Wearables that sense and change shape in respond to humidity	Body sweat (15 - 25% relative humidity)	Fluorescence expression (GFP)	[157]
Saccharomyces cerevisiae	Alginate/paper	Detection of antibiotics	Doxycycline (0.3 - 100 µg/mL)	Fluorescence expression (RFP)	[169]
Escherichia coli	Polyacrylamide/alginate	Detection of blood	Heme from defibrinated horse blood (not specified)	Bioluminescence	[170]
Escherichia coli	Polydimethylsiloxane, parylene/epoxy, microelectronics	Monitoring gut biomarkers	Heme (32.5 - 500 ppm), N-acyl homoserine lactone (100 nM), and thiosulfate (10 mM)	Bioluminescence	[171]

[a] GFP: green fluorescent protein. RFP: red fluorescent protein

the matrix but appeared to be slower than the production obtained from freely suspended cells. The inclusion of quorum sensing molecules to the media, such as acylated homoserine lactones (5 μM), appeared to enhance viability and increase prodigiosin production by 20% more than the production obtained in the absence of these molecules.[196] In another report, probiotic bacteria, *E. coli* Nissle 1917, was transformed to encode GFP and to secrete model proteins. Cells were entrapped in electrospun polyethylene glycol-polylactide porous fibers and further immobilized by adsorption or covalent binding to the surface of the fibers.[198] These porous fibers allow sufficient nutrient diffusion for cell growth and protein secretion when exposed to chemical stimuli. ELMs were incubated in the presence of the chemical inducer IPTG (1 mM) to induce cell proliferation. The structures were capable of undergoing fluorescence by expression of intracellular GFP and also secrete the proteins into media.[198] This strategy serves as a proof-of-concept approach for the development of drug delivery devices that use probiotics for the potential release of therapeutic drugs in the body. Another drug delivery approach utilized light to spatially and temporarily control intracellular drug production in bacteria. This study made use of an endotoxin-free *E. coli* strain encapsulated in agarose hydrogels.[63] This strain was programmed to express a *vioABCE* operon for the metabolic production of deoxyviolacein (dVio), a bacterial metabolite with anti-bacterial, anti-fungal, and anti-tumor activities.[199,200] The *vioABCE* operon was placed next to the optogenetic plasmid pDawn, to build a genetic circuit with light-responsive capabilities for the production of dVio (Fig. 6A). It was found that, when the ELM was exposed locally to blue light, *in situ* production of dVio was obtained (Fig. 6B).[63] In addition, drug production led to a change in color of the material to a dark purple and the development of weak fluorescence when the material was exposed to light intensities

of 1.38 mW/cm^2 (Fig. 6A). Nevertheless, the drug remained in the bacterial cytosol and was only released when a nonionic surfactant was provided to the hybrid material.[63] Similar to this approach, the same group developed an ELM using *E. coli* encapsulated in agarose gels that delivered a protein in response to blue light.[201] Bacteria were constructed with a streptadivin-binding peptide containing RFP. The ELM was capable of expressing the RFP protein within the gel and secrete it to the surrounding growth media using blue light. Fluorescence changes within the ELM were observed, and protein secretion of around 40 ng per 10 μL of bacterial hydrogel was obtained within a day of pulse-cycle light illumination.[201] In the next approach, *E. coli* was engineered to produce a cell-adhesive protein on the bacterial surface.[202]

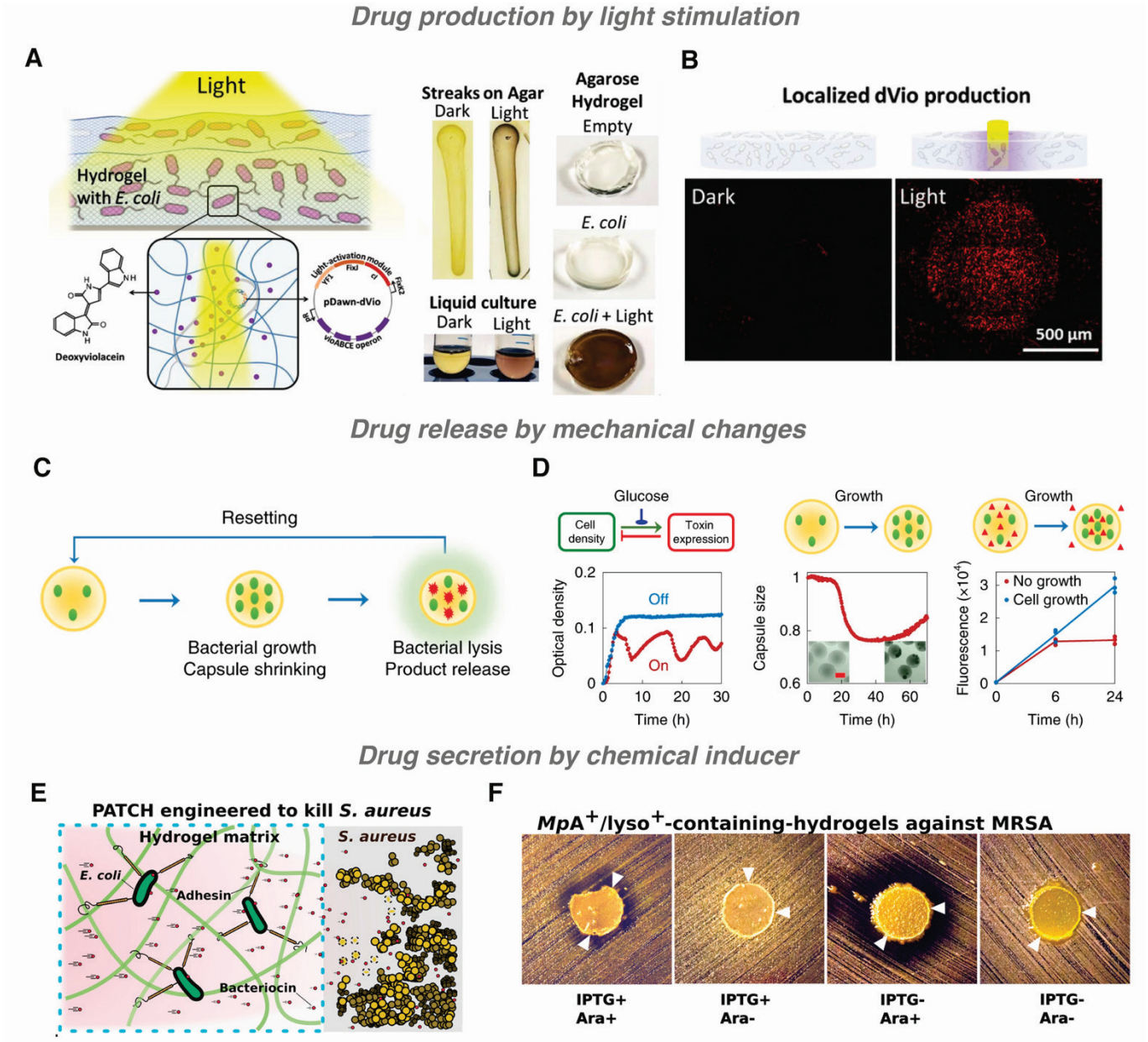

Fig. 6 Stimuli-responsive ELMs for drug delivery applications. (A) Schematic of a light-responsive ELM. Bacteria was engineered with a pDawn-dVio plasmid to enable the production of the drug deoxyviolacein upon light illumination (left). Bacterial cells cultured on agar, liquid media or encapsulated in agarose hydrogels were capable of producing the drug and a change in color in response to light (right). (B) Patterned light exposure allowed the production of drug locally and was detected by epifluorescence microscopy. Adapted with permission from ref. (63). Copyright (2018) Wiley-VCH. (C) Schematic representation of beads encapsulating bacteria that shrink in response to cell growth and swell in a reversible manner to enable an oscillatory behavior. Bacteria undergo lysis at sufficiently high cell densities and then release model molecules to the surroundings. (D) Oscillatory behavior can be turned off by adding glucose to the media. Capsules shrink in response to cell growth. Capsules that shrink lead to an increase in fluorescence intensity as compared to capsules that do not shrink. Adapted with permission from ref. (206). Copyright (2019) Wiley-VCH. (E) Schematic of the ELM encapsulating engineered bacteria that produce and secrete an antimicrobial enzyme against methicillin-resistant *S. aureus*. (F) Antimicrobial activity of ELMs placed on Mueller-Hinton agar plates streaked with methicillin-resistant *S. aureus*. Adhesin and lysostaphin functionalities by cultivating ELMs in the absence or presence of IPTG and/or Arabinose. Adapted with permission from ref. (207). Copyright (2020) American Chemical Society.

Bacteria were engineered to express an RGD adhesive miniprotein along with a red fluorescent protein when sensing the presence of a photoactivatable version of IPTG (PA-IPTG). Bacteria were immobilized on poly-D-Lysine coated nexterion slides and induced with PA-IPTG and a light source. This stimulus activated miniprotein expression in the cell membrane and the production of RFP. To study the presence of the miniprotein produced, mouse embryonic fibroblasts were seeded on the materials. These fibroblasts displayed integrins that interacted with the proteins and adhered to the ELM. Additionally, it was observed that RFP could eventually secrete outside of the bacteria, which opens up opportunities to target drug delivery in mammalian cells.[202] These approaches enable the specific control of light for spatio-temporal control of bacterial metabolic functions for drug production. Further studies could be investigated towards the implementation of material processing techniques that allow better diffusion of nutrients and penetration of light for secretion of drugs.

The design of materials that actively secrete drugs to the surroundings are highly desired. To create drug-releasing ELMs, an antibiotic-producing fungus, *Penicillium chrysogenum*, was encapsulated in a sandwich-like structure for the production of penicillin. The structure consisted of a nanoporous top layer for diffusion of nutrients, a middle layer made of agar and the living component, and a bottom layer for mechanical support. The resulting ELMs were capable of maintaining sustained release of penicillin for days when the materials were provided with sufficient external nutrients. The nutrients diffused through the top layer and allowed the fungi to grow and release penicillin on top of the ELM surface. Further, the material could effectively inhibit the growth of penicillin-sensitive *Staphylococcus carnosus* within one day.[203] In other studies, the use of bacteria, such as *Lactococcus lactis*, was harnessed to control the behavior of human mesenchymal stem cells (hMSCs) upon addition of peptides, such as nisin.[204] Bacteria were engineered to express human fibronectin to support stem cell adhesion and to secrete a bone morphogenic protein (BMP-2) that induces osteogenic differentiation in hMSCs. Addition of nisin as an external stimulus promoted fibronectin expression on the bacterial cell membrane and the secretion of BMP-2 to the extracellular environment. To build ELMs, engineered bacteria were cultured on different materials, such as poly(ethyl acrylate) or collagen, to allow biofilm formation and adherence to the substrate. ELM function was tested using different concentrations of nisin. After adding 10 ng/mL of this molecule to the ELM, fibronectin expression was induced, and subsequent hMSC adhesion was obtained. In addition, bacterial cells were capable of secreting high concentrations of BMP-2 (200 ng/mL) that contributed to osteogenic differentiation of hMSCs.[204] These stimuli-responsive ELMs are suitable for delivery of proteins to the surrounding environment and enable strategies that can be used for stem cell research or drug-delivery platforms. The same group utilized a droplet microfluidic device to construct compartmentalized microgels that encapsulated both of the previously described bacteria and hMSCs within the same structure. The microgels served as extracellular matrices to study osteogenesis upon addition of extracellular nisin.[205] This versatile encapsulation platform enables strategies to study drug production and symbiotic cellular interactions. An approach that utilized the proliferation of living microorganisms to build oscillatory platforms for controlled release of model proteins has been investigated.[206] This strategy utilized pH-responsive chitosan capsules containing genetically engineered *E. coli* built with a genetic circuit that causes oscillations in cell density over time (Fig. 6C). By introducing nutrients as external cues, bacteria were capable of proliferating and undergoing partial lysis by expressing toxins upon reaching sufficiently high cell densities within the capsule. Accumulation of toxins caused cell lysis and a decrease in cell density, but partial decrease allowed growth recovery and, therefore, the generation of growth oscillations for multiple cycles. Cell proliferation and subsequent lysis led to a change in pH of the surrounding media and, eventually, the shrinkage of the chitosan capsules to squeeze out bacterial lysis protein products. Swelling was observed by controlling the lysis rate after shrinking when glucose was added, and by replenishing the capsules with fresh media periodically. The model protein released from the capsules was β-lactamase, and its presence in the surrounding media was detected by adding a substrate to measure enzymatic activity (Fig. 6D).[206] This ELM platform demonstrates the active feedback control that can be mediated by the interactions between engineered cells and stimuli-responsive materials to create drug delivery platforms. Genetically-engineered *E. coli* with cell surface-displayed adhesin proteins and triggered drug secretion enable multifunctional ELMs. The bacteria were able to be retained in dextran-based hydrogels by displaying a calcium-dependent, glucose-binding adhesin. The adhesin protein (*Mp*A), derived from Antarctic bacterium *Marinomonas primoryensis* binding proteins, was expressed on the surface of the cells. When the chemical IPTG was present, the protein could bind glucose with high affinity. Additionally, to create a stimuli-responsive ELM, cells were also transformed to express and secrete bacteriocin lysostaphin. This molecule was secreted to target and inhibit the growth of pathogenic bacteria, *Staphylococcus aureus*, when the ELM was exposed to the monosaccharide arabinose (Fig. 6E). The results showed that upon exposure to arabinose, the device was activated for the in situ secretion of lysostaphin, which diffuses out of the cells and inhibits the growth of methicillin-resistant *S. aureus*.[207] The bactericidal activity was tested when the hydrogels were placed on agar plates streaked with *S. aureus*, in the absence or presence of IPTG and/or arabinose. Bactericidal activity against *S. aureus* was induced by adding arabinose and observed by the development of inhibition zones around the periphery of the ELMs (Fig. 6F).[207] This work demonstrates the application of an engineered living material with specific responses to biomolecules for drug secretion and enables the design of devices for therapeutic applications. Further work could be focused on the implementation of 3D printing to build structures that allow higher cell biomass retention and that improve the efficient secretion of drugs. An overview of the applications described in this section is given in Table 3.

Table 3 Stimuli-responsive engineered living materials for drug-delivery applications

Biological component	Synthetic material	Function	Stimulus	Response[a]	Ref.
Serratia marscescens	Silica	Drug production of prodigiosin	Trypto casein soy media, N-butanoyl-l-homoserine lactone (5 μM) or N-hexanoyl-l-homoserine lactone (5 μM).	Production of prodigiosin within the matrix	[196]
Escherichia coli	Polyethylene glycol-polylactide porous fibers	Secretion of GFP to the surrounding media	Isopropyl β-D-1-thiogalactopyranoside (1 mM)	Fluorescence expression and secretion of GFP	[198]
Escherichia coli	Agarose or polyacrylamide	Drug production of deoxyviolacein	Blue light (1.38 mW/cm² and 470 nm)	Production of deoxyviolacein and fluorescence expression of RFP	[63]
Escherichia coli	Agarose	Protein production	Blue light (12.5 mW/cm² and 470 nm)	Production and fluorescence expression of RFP	[201]
Escherichia coli	Poly-D-Lysine coated nexterion slides	Protein production and mammalian cell adherence	Photoactivatable Isopropyl β-D-1-thiogalactopyranoside (500 nM)	Fluorescence expression and production of RFP, production of adhesive protein	[202]
Penicillium chrysogenum	Agarose, nanoporous polycarbonate layer and mechanical support	Production of penicillin and antimicrobial function	Culture media rich in sugars glucose and galactose	Production of penicillin outside the materials and inhibition of penicillin-sensitive *Staphylococcus carnosus*	[203]
Lactococcus lactis	Poly(ethyl acrylate), collagen	Control the behavior of human mesenchymal stem cells	Nisin (10 ng/mL)	Production of fibronectin to allow stem cell adherence and production of bone morphogenic protein to allow osteogenic differentiation	[205]
Escherichia coli	Chitosan	Bacteria grows in an oscillatory form to for change shape and the release of β-lactamase	Nutrient media	Bacteria grows and undergoes lysis which change the surrounding pH. pH-Chitosan shrinks and swells to release bacterial β-lactamase	[206]
Escherichia coli	Dextran	Adhesin production and secretion of microbial bacteriocin	Isopropyl β-D-1-thiogalactopyranoside (0.5 mM) and arabinose (5 mM)	Bacteria adheres to the material and secretes lysostaphin to inhibit the growth of *S. aureus*	[207]

[a]GFP: green fluorescent protein. RFP: red fluorescent protein

3.2.2.1 Ongoing challenges and future directions

Stimuli-responsive ELMs could facilitate long-term delivery of therapeutics that may be impossible to obtain with traditional or purely synthetic stimuli-responsive materials. The key advantages of using ELMs are that a variety of stimuli can initiate drug delivery and that the drug could be synthesized within the ELM. As described in this section, production of model drugs and therapeutics from ELMs usually involves the

use of engineered microorganisms. In these studies, cells are modified to express heterologous genes that encode a molecule of interest only when an exogenous stimulus is applied. This enables the targeted delivery of therapeutics only when and where required. Nevertheless, the release of some drugs to the surroundings using ELMs is a key challenge. Some drugs that are synthesized within the cells require exportation through the cell membrane and must diffuse out of the ELM. Exportation is often limited to small molecules that may be able to cross the microbial cell membrane freely. However, for larger molecules this exportation usually requires the engineering of cells with appropriate secretion systems. These systems can have limited capacity and become saturated, leading to drug retention. Alternative strategies involve cells programmed to lyse to release their cargo. In addition, when building stimuli-responsive ELMs, the encapsulating matrices could be designed to further modulate diffusion of therapeutics out of the material. Another key advantage of these materials is their use as gut therapeutics. ELMs could be used to detect relevant biomarkers found in the gastrointestinal tract and control targeted drug delivery. As described previously, ELMs have been reported to sense disease related gut biomarkers, but the integration of a therapeutic approach in this area remains to be studied. We expect that drug delivery from ELMs may be most compelling when the properties of both the living cell and the synthetic material are used to control the release profile.

3.2.3 Soft robotics

Engineered living materials with the ability to sense their environment and respond mechanically are of great interest for the fabrication of soft biohybrid robots. Soft robots are capable of performing functions for healthcare applications, benefiting from their ability to adapt, undergo complex motions, and increase compatibility with the mechanics of the body.[208,209] Soft robots that are made of stimuli-responsive soft materials, including electroactive polymers,[210] pneumatic elastomers,[211] shape memory polymers,[212,213] liquid crystal elastomers,[214,215] or hydrogels[216,217] may enable untethered systems with sensing and shape-changing abilities. Each of these materials requires strong, external power sources to change shape, and the delivery of this power, such as by heating the material, may be incompatible with the environment surrounding the robot.[218,219] In this context, engineered living cells enable soft robots that are, at least partially, chemically powered by the metabolism of the cells. So far, we have described on ELMs mainly composed of microorganisms, which provide exceptional control mechanisms for creating stimuli-responsive ELMs with the advances of synthetic biology tools. Mammalian cells offer unique capabilities, including coordinated and synchronized actuation for the design of soft robots. The pioneering work that placed the foundation for building soft biohybrid robots utilized rat ventricular cardiomyocytes seeded on polydimethylsiloxane (PDMS) to create muscular thin films. Upon exposure to electrical stimuli, cells underwent synchronized contraction and relaxation that caused the underlying films to transform from 2D to 3D shapes, and perform functions such as gripping, pumping, walking, and swimming.[220] Building on this work, cardiomyocytes were used to fabricate jellyfish-like soft robots that achieve complex swimming behavior.[221] Other research studies that take advantage of contraction and relaxation properties of cells utilized a range of muscle cell types, including skeletal muscle myotubes, smooth muscle cells, stem cell-derived cardiomyocytes, among others.[222–225] The ability of cells to respond to chemicals in the environment has also allowed the fabrication of color-changing biosensors with actuating capabilities powered by cardiomyocytes.[226,227] In one example, neonatal rat ventricular cardiomyocytes have been cultured and oriented on *Morpho menelaus* butterfly wings to function as beating components and cause structural color shifts. Upon addition of isoproterenol, cardiomyocytes were stimulated to increase beating frequency, which resulted in an increase in the degree of structural color changes. Studies in this field offer advances for creating self-reporting platforms to evaluate drug effects on mammalian cells.[227] The biohybrid methods described so far are able to function at high energy efficiencies and harvest energy from surrounding nutrient solutions.

Optogenetics enables the realization of soft biohybrid robots with fast and precise control over multiple muscle units, necessary to power locomotion and achieve coordinated robotic maneuvering. Cardiac and skeletal muscle cells have been modified to express light-gated ion channels, Channelrhodopsin-2, to create blue-light-sensitive constructs.[62,228–230] An interesting approach, where cardiac cells were patterned onto a four-layered architecture, yielded a tissue-engineered artificial stingray with phototactic control on sequential muscle cell activation for undulatory locomotion (Fig. 7A).[228] The stingray was guided along an obstacle course by modulating the frequency of the applied light and controlling directional turns (Fig. 7B). Another biohybrid robot based on optogenetics utilized skeletal muscle cells to fabricate bioactuators with 2D directional locomotion and rotational steering in response to both electrical and optical stimulation.[229] Further, the same group optimized the system by developing strategies to drive healing and remodeling after mechanical damages.[230] These approaches are promising for creating soft robots, but the maintenance of mammalian cell cultures needs to be carefully controlled. As a result, these devices are highly unlikely to persist for long periods of time in various environments.

A strategy that developed a method to actuate a skeletal muscle cell-based biohybrid robot in air has been described.[231] Collagen hydrogel and a system of tubes to perfuse culture medium were used to encapsulate skeletal muscle tissue and maintain the necessary humidity conditions for cell viability. Electrical stimulation was applied through embedded electrodes to induce tissue contractility when the robot was operated in the air (Fig. 7C). This stimulation allowed the control of the deformation of the biohybrid robot and was demonstrated by pushing a bead in air (Fig. 7D). Although this system gradually dries out, its contractility function was continuously maintained

for 1 h without any damage to the skeletal muscle tissue.[231] Insect muscle cells have relatively longer-term viability and can tolerate fluctuating changes in temperature, pH, or oxygen, and thus, they have been studied to create robust soft robotic actuators.[232,233] In addition, the use of motile microorganisms expands on the use of living cells to create microrobots that have better stability in a wide range of environmental conditions and that can be genetically modified to complete complex functions. Microrobots have been described in soft robotic reviews where motile bacteria, microalgae, and mammalian cells sense the environment and are manipulated by external stimuli. Their fabrication is of potential use in

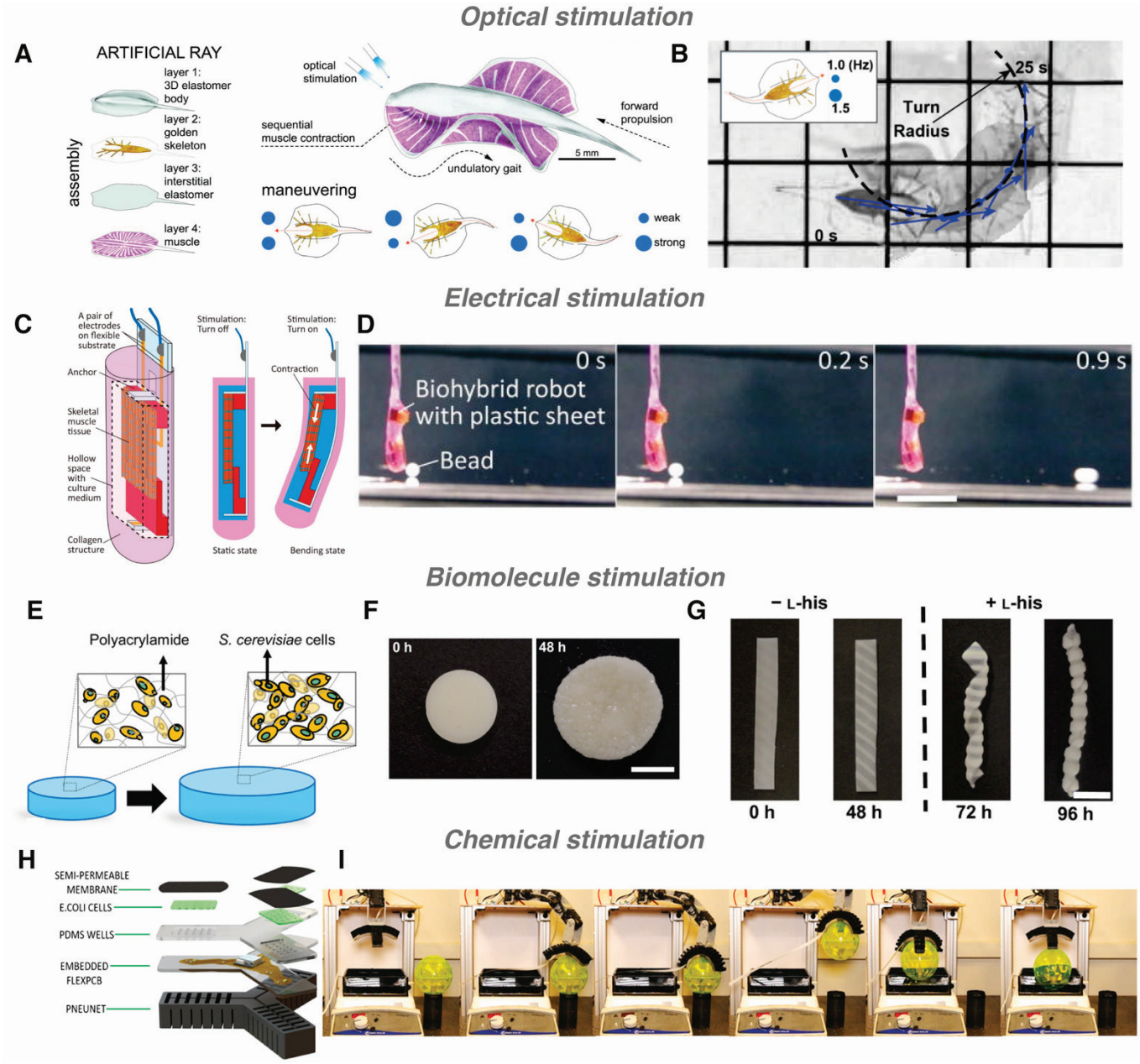

Fig. 7 Soft biohybrid robots actuated by external stimulation. (A) Soft robotic ray composed of genetically engineered cardiomyocytes in an elastomeric body. The robot was controlled by optical stimulation to induce sequential muscle cell activation and locomotion. (B) Asynchronously triggering modulated by light frequency with 1.0/1.5 Hz paired pulses resulted in directional turns. Adapted with permission from ref. (228). Copyright (2016) American Association for the Advancement of Science. (C) Biohybrid robot composed of skeletal muscle tissue encapsulated in collagen structure. Upon applied electrical stimulation, the biohybrid robot actuates in air. (D) Motion control was demonstrated by pushing a bead through deformation of the collagen structure from muscle contractions (Scale bar: 1 cm). Adapted with the permission from ref. (231). Copyright Yuya Morimoto, Hiroaki Onoe, Shoji Takeuchi, APL Bioengineering, Vol.4, https://doi.org/10.1063/1.5127204, 2020; licensed under a Creative Commons Attribution (CC BY) license. (E) Schematic representation of a shape-morphing living composite that changes in volume in response to yeast proliferation. (F) Living composite before and after incubation shows an increase in volume (Scale bar: 7 mm). (G) UV-patterned film remains inactive when L-histidine is not provided and changes into a helical structure when L-histidine is detected by the encapsulated yeast (Scale bar: 1cm). Adapted with permission from ref. (240). Copyright (2020) exclusive licensee American Association for the Advancement of Science. Distributed under a Creative Commons Attribution License 4.0 (CC BY) http://creativecommons.org/licenses/by/4.0/. (H) Components used to build a soft biohybrid gripper with embedded genetically engineered bacterial cells. (I) When the gripper was found to not contain the chemical inducer IPTG, the robotic arm was capable of picking up an object and placing it into the media bath. Adapted with permission from ref. (244). Copyright (2019) American Association for the Advancement of Science.

applications for specific targeting and delivering of cargo or for powering 3D micromotors.[234–236] Nevertheless, these types of devices are only manipulated for their use at the microscale, due to the fixed size of the biological units. Methods that focus on the sensing and responsive capabilities of microorganisms to drive actuation in macroscale soft robots, by applying external stimuli, are also of interest in this review.

Many materials in nature undergo shape transformations in respond to environmental conditions such as humidity. Hygromorphic materials have been studied to produce actuators that operate by changes in moisture found in the skin or the air. Studies that use the hygroscopic properties of natural cells, to convert energy from humidity gradients have made use of bacterial spores as building blocks for the design of macroscopic actuators.[237] Bacterial spores from *B. subtilis* possess a cortex that is hygromorphic, have long term survivability, and do not require the addition of a nutrient source to maintain its hygromorphic behavior.[238] These spores have been applied in the fabrication of stimuli-responsive ELMs as they can reversibly expand or shrink to changes in relative humidity. For example, in one study 3 mg of these spores were coated on 0.5 mm or 0.75 mm thick latex sheets treated with poly-L-lysine to improve adhesion.[237] After fabrication, the ELMs have an initial curvature at a relative humidity of 15-20% from laboratory conditions. This curvature starts decreasing when the ELM is subjected to increasing relative humidity. The ELMs have a fast, reversible response because the spores respond mechanically within ~0.4 s after exposure in humid conditions and within ~0.5 s of water release. Because of these unique responses, ELMs were applied to harvest energy, and it was observed that the spores delivered an average power of 0.7 μW with an estimate of ~233 mW/kg for electrical power.[237] These materials were further studied to create ELMs that undergo complex transformations. Bioprinting techniques were used to spatially localize *B. subtilis* spores on flat substrates.[238] These materials were capable of undergoing complex 3D folding in response to relative changes in humidity. Another report utilized the same bacterial spores to build structures using photolithography and UV-curable resins.[239] Spores were mixed within the resin and then UV-cured on top of a polyimide substrate to build the devices. Further, the polyimide substrates were laser cut and patterned with the spore/UV-resin solution through photolithographic masks. This design allowed ELM actuators to undergo complex changes in shape under relative humidity conditions. These materials presented work and power intensities at a maximum of 0.44 MJ/m^3 and 54 kW/m^3, respectively.[239] These hygromorphic ELMs could be used in future applications for energy harvesting and soft robotics.

Yeast proliferation within polymer materials has been harnessed to drive mechanical actuation in macroscopic structures. Baker's yeast, *S. cerevisiae*, encapsulated in polyacrylamide hydrogels, were capable of proliferating within the matrix when essential nutrients were provided (Fig. 7E).[240] Cell proliferation led to the material expanding its volume up to 200% more than when nutrients were absent (Fig. 7F). Genetic engineering of the yeast enabled high control of shape-change by using specific biogenic amines, such as L-histidine, that triggered cell proliferation within the composite. The engineered yeast could sense external stimuli and respond by irreversible expanding the polymeric matrix. By patterning cell viability, complex structures such as helical geometries could be obtained (Fig. 7G). Optogenetic switches were also engineered in the yeast and enabled the spatiotemporal control of ELM actuation when the materials were exposed to a light intensity of 2.7 mW/cm^2.[240] We note that this approach utilized visible light at a weak irradiation intensity to induce shape change in the ELM. Many strategies in traditional stimuli-responsive materials, built to change shape, require UV or visible light at strong irradiation intensities of 100 mW/cm^2 or higher.[241,242] High intensities may cause overheating and undesired health effects, such as tissue damage when ELMs are used for biomedical applications. However, it is important to note that parameters such as irradiation time and wavelength of light need to be considered when making this comparison.[243] A method that developed a soft robotic gripper utilized an interfacial module that allowed the communication between living bacteria, the external environment, and electronics embedded in soft materials.[244] The interface module allowed the biocontainment of engineered bacteria to detect environmental signals that then where converted into cellular and to electronic signals. The soft robotic gripper consisted of *E. coli* carrying the plasmid *pIV_GFP* to allow the synthesis of GFP in the presence of chemical inducer IPTG. Cells were cultured, retained by membranes, and housed within a PDMS chamber to create a biolayer. Then, a flexible printed circuit board (FlexPCB) and pneu-nets were combined with the biolayer and mounted to a 4-DOF robotic arm (Fig. 7H). This device was designed to produce a fluorescent signal in response to IPTG, allowing the electronic components to detect the signal and distribute it to a central processing unit to initiate robotic decision making and actuation. To initiate the process, the device was incubated in the absence or presence of IPTG and allowed to measure and store the data of the cell fluorescent output. After incubation, the gripper 'decides' whether or not to deploy a round object into the media. When the device detects the presence of IPTG, it alerts the system and does not deploy the object. In the absence of IPTG, the system then decides that it is safe to grab and deploy the object (Fig. 7I).[244] This approach demonstrates the possibility of combining genetically engineered living microorganisms, electronics, and soft materials to create a responsive, actuating device that communicates with the external environment and excels at a decision-making process. An overview of the applications described in this section is given in Table 4.

Table 4 Stimuli-responsive engineered living materials for soft robotic applications

Biological component	Synthetic material	Function	Stimulus	Response	Ref.
Rat ventricular cardiomyocytes	Polydimethylsiloxane	Contraction and relaxation of polymeric sheets	Electrical field (10 V, 10 ms pulse-width and frequency 0.25 - 5 Hz)	Reversible actuation such as gripping, pumping, walking, and swimming	[220]
Neonatal rat ventricular cardiomyocytes	*Morpho menelaus wings, carbon nanotubes, methacrylated gelatin*	Contraction and relaxation of morpho wings to shift structural colors	Isoproterenol (1 μM)	Reversible actuation, structural color shifts	[227]
Rat cardiomyocytes	Polydimethylsiloxane	Muscle cell activation for undulatory locomotion	Blue light (0.3 - 12 mW, 300 ms pulse light, and 470 nm)	Soft robotic actuation and locomotion	[228]
Skeletal muscle tissue	Collagen, perfusion tubes	Actuation in air	Electrical pulses (1 Hz and 50 Hz frequency, 2 ms pulse duration)	Soft robotic actuation in air	[231]
Bacillus subtilis	Latex sheets	Hygromorphic actuation	Relative humidity changes (15 - 90 %)	Fast, reversible actuation in response to humidity changes	[237]
Bacillus subtilis	UV-curable resins, polyimide	Hygromorphic actuation of complex structures	Relative humidity changes (10 - 90 %)	Actuation in response to humidity changes	[239]
Saccharomyces cerevisiae	Polyacrylamide	Shape change in response to biogenic amines and light	Amino acid (L-histidine) and low power blue light (2.7 mW/cm²)	Cellular proliferation induces shape transformation	[240]
Escherichia coli	Polydimethylsiloxane, flexible printed circuits, pneu-nets, semipermeable membrane	Communication between cells, the external environment and electronics to activate soft gripper actuation	Isopropyl β-D-1-thiogalactopyranoside (0 or 1 mM)	Activation of the soft robotic gripper in the absence of the stimulus	[244]

3.2.3.1 Ongoing challenges and future directions

Within this section, we described using the mechanical nature of living cells that respond to an external stimulus to drive ELM mechanical deformation. The key advantages of using living cells to control or drive mechanical motion include that the device is chemically powered and can respond to weak and poorly-differentiated stimuli. However, further work is required in a number of areas regarding ELM-based soft robots. Durability is one key challenge. At one end of the spectrum, hygromorphic bacterial spores in fabrics use only the biophysical characteristics of the spores and do not involve processes at the cellular level. This limits the number of stimuli that can be used, but the ELMs should be quite durable. By contrast, in studies involving the use of mammalian muscle cells, the cellular environment, from fabrication through use, needs to be carefully controlled to ensure functionality of the device. The speed of response is another key challenge. Muscle-based or hygromorphic spore-based ELMs can respond on the order of seconds. Actuators based on cellular proliferation are slow which limits their use to applications that do not require rapid or reversible motion. We expect that living cells will continue to be used to control traditional actuators and to serve directly as actuators in applications where chemical powering of the robot is critical, such as in small, untethered, and implanted robots, and in applications where autonomous response to biochemical or weak physical cues is desired.

3.3 Other applications of stimuli-responsive ELMs

Stimuli-responsive ELMs have also been fabricated that perform self-healing and self-cleaning functions. For example, in civil engineering applications, where concrete structures are susceptible to cracking due to natural processes, ELMs have been fabricated to utilize living bacteria to repair cracks. Several strategies have been described that utilize bacteria as a self-healing agent capable of surviving the harsh environment of concrete mixing.[245–250] These strategies typically utilize concrete matrices mixed with immobilized bacterial strains. Bacteria are capable of converting calcium lactate into calcium carbonate by using external oxygen. Calcium carbonate acts as a material that precipitates in cracks and heals concrete. Different carriers for immobilization of bacteria have been used, such as clay aggregates, perlite, microcapsules, and super absorbent polymers.[245,251–254] For example, spore-forming *B. subtilis* was genetically transformed with a biosilicification gene, BKH2, and incorporated in concrete/mortar matrices. The matrices were subjected to loads to create micro/macroscopic cracks and then immersed in growth media to start the healing process. Due to biochemical activity of the engineered cells, nano-rod sized gehlenite along with calcite were developed within the cracks of the concrete matrices after several days.[255] The performance of the material significantly improved in its self-healing property, mechanical strength, and durability, as compared to previous studies.[256–258] Another strategy utilized plant cell chloroplasts, glucose oxidase, and a monomer to create self-healing hydrogel matrices.[259] The chloroplasts were extracted from spinach and were used as carbon-fixing photocatalysts that utilize solar energy and atmospheric carbon dioxide to produce glucose. The chloroplasts and glucose oxidase were embedded in lightly crosslinked hydrogel networks capable of swelling, growing, and self-healing themselves. When the gels were illuminated with light and provided CO_2, the glucose produced reacted with glucose oxidase and was converted into gluconolactone (GL). The monomer was capable of undergoing polymerization by reacting with GL. The self-healing capability was demonstrated in physically separated hydrogels that recombined and recovered their mechanical strength when illuminated with light.[259] These ELM strategies are conceptually related to self-healing polymers that repair themselves upon crack formation. Some examples of these strategies employ dual-microcapsule systems that contain a healing agent and catalyst.[260,261] When cracks form, these materials release a healing agent that cures upon contact with the released catalyst to then heal the damaged areas. However, they require catalysts to be readily available, can generate toxicity, and require homogenous distributions of both components.[262]

To create ELMs with self-cleaning properties, a report that developed a 'living surface', based on living *Penicillium roqueforti,* was used to metabolize model food spills. The living surface consisted of three layers: a bottom layer for support, a middle layer that encapsulated the living cells and a top layer that protected the living component from the environment. The ELM remained dormant until exposure to the food spill. When a food spill was detected on the surface, the living component consumed the food and proliferated within the material, therefore, creating a self-cleaning behavior.[263] Even though this strategy utilized model food spills (e.g. glucose containing droplets) to test the self-cleaning performance, the living components can be further designed to metabolize other components or produce antimicrobial molecules to generate self-sterilizing surfaces. Similar strategies that utilize synthetic materials with self-cleaning properties have been designed to respond to external stimuli to change surface wettability.[264] Examples include materials that switch between superhydrophilicity and superhydrophobicity states in response to pH, temperature or light.[265,266] These strategies do not metabolize target molecules but prevent the surfaces from adhesion of contaminants, for example, by removing dirt or oil. Nevertheless, these strategies need to overcome limitations such as fouling of the material's surface by oils and poor recyclability, which can reduce their self-cleaning ability and their implementation in practical applications.[267,268]

4. Conclusions

Within this review, we have described materials with programmed functions governed by living cells, where responses to external cues have enabled biosensors, analytical and monitoring devices, drug delivery platforms, soft biohybrid robots, and materials with self-healing or self-cleaning capabilities. The responses obtained from the living component are observed when the whole material undergoes physical or chemical changes such as fluorescence, luminescence, production of molecules, enzymatic activity, color changes, and shape transformations. These promising advances broaden possible engineering technologies for environmental remediation where specific detection, degradation, or monitoring of pollutants in the environment have the potential of recycling and managing hazardous wastes. For biomedical applications, stimuli-responsive ELMs enable biosensors for the detection and monitoring of disease-related biomarkers, drug delivery devices for the production of therapeutic molecules at specific target sites, and soft robots. With the growing fields of synthetic biology and soft materials science, we expect that stimuli-responsive ELMs will rapidly show significant developments in programmable active matter.

Conflicts of interest

There are no conflicts to declare.

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Engineered living materials integrate genetic engineering and synthetic materials to program stimuli responses that enable the fabrication of devices for diverse applications.