Engineered living biomaterials

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Abstract | Biomaterials have evolved from inert materials that lack interaction with the body to biologically active, instructive materials that host and provide signals to surrounding cells and tissues. Engineered living materials contain living cells (responsive function) and polymeric matrices (scaffolding function) and, thus, can be designed as active and response biomaterials. In this Review, we discuss engineered living materials that incorporate microorganisms as the living, bioactive component. Microorganisms can provide complex responses to environmental stimuli, and they can be genetically engineered to allow user control over responses and integration of numerous inputs. The engineered microorganisms can either generate their own matrix, such as in biofilms, or they can be incorporated in matrices using various technologies, such as coating, 3D printing, spinning and microencapsulation. We highlight biomedical applications of such engineered living materials, including biosensing, wound healing, stem-cell-based tissue engineering and drug delivery, and provide an outlook to the challenges and future applications of engineered living materials.

Living cells have the ability to synthesize complex molecules from a few precursors, which can assemble and grow into materials with impressive performance and complexity in composition and structure[1]. Natural materials, such as bone, wood or bacterial biofilms, can further adapt their composition, structure and performance in response to changes in environmental factors, such as molecular effectors, pH, salinity, temperature, light intensity or direction, or mechanical stress[2–4]. Autonomous growth, sensing, secretion of metabolites and regeneration are inherent to many natural living materials. The growing field of engineered living materials aims at recreating these properties of living materials for the design of functional and responsive materials[5].

A variety of organisms, including bacteria, fungi, algae and animal cells, have been incorporated into materials, such as silk, concrete or hydrogels, to engineer living materials, or biohybrids, for a range of applications[6–10]. In particular, synthetic biology has enabled the precise genetic engineering of cells for biotechnological applications, including cell programming and gene regulation. Engineered living materials for biomedical applications have emerged from combining these synthetic biology capabilities with advances in bioengineering and tissue engineering concepts[5,7]. In living materials, living cells are combined with a matrix or scaffold. The living component endows the material with distinct functional properties. Living materials can be generated by a top-down approach, based on the design of a non-living composite that encapsulates living cells. Alternatively, in a bottom-up approach, cells can be engineered to synthesize and maintain the surrounding matrix, such as in engineered biofilms[11–13].

Engineered living materials are particularly interesting for biomedical applications; for example, wearable biosensors can be designed by incorporating engineered microbial strains that produce output signals, such as the expression of a fluorescent protein, in response to contaminating biochemicals[14]. Such functionalities are achieved by integrating synthetic genetic circuits[15,16] and engineered sensory proteins[17,18] with the biosynthesis of proteins or small molecules. The field of drug delivery aims at delivering therapeutic molecules to specific sites in the body, to optimize the pharmacokinetic profile of the drug[19–22]; for example, growth factor-loaded sponges can be implanted to promote bone tissue regeneration, such as InFuse, which is a collagen sponge delivering bone morphogenetic protein 2 (BMP2) for spinal fusion. Alternatively, a growth factor-eluting engineered biofilm can continuously produce the desired dose of a therapeutic agent at the regeneration site to stimulate stem cell differentiation and tissue regeneration[23]. Similar therapeutic biofilms and drug-delivering living implants have been explored, including skin patches for wound healing[24,25], adhesive bacterial matrices for the treatment of chronic inflammation in the intestine[26] or to seal blood leakage in vascular tissues[27], and self-replenishing drug depots for the delivery of antimicrobials or therapeutic proteins[24,28–31] (FIG. 1). Most living materials are based on laboratory strains of *Escherichia coli*, because *E. coli* can be easily genetically modified. However, biomedical applications would require the use of Generally

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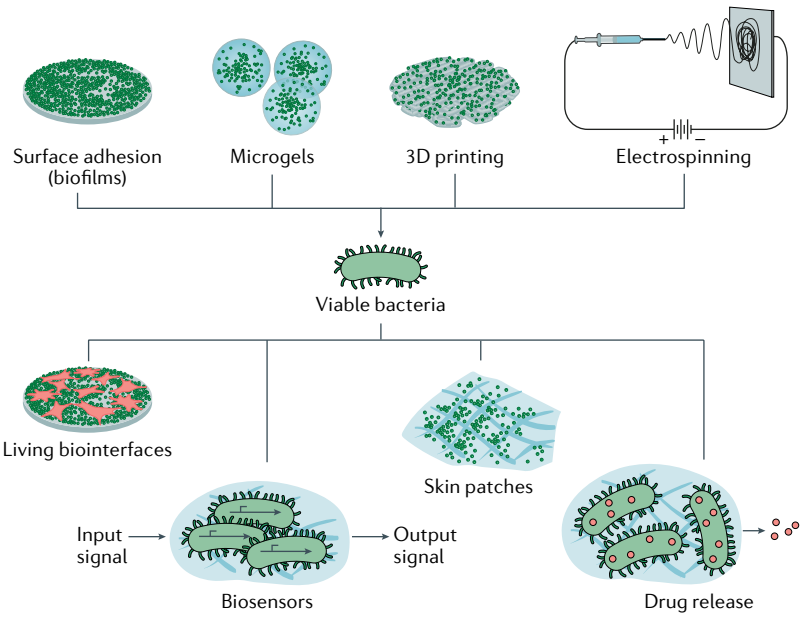

Fig. 1 | **Fabrication and biomedical applications of living materials.** Living materials contain wild-type or engineered bacteria that respond to external stimuli and are incorporated on the surface or within material substrates, which provide structure and support. Living materials can be fabricated by surface engineering, allowing the development of biofilms, by encapsulation into hydrogels to form microgels, which can be used as self-contained microreactors, by 3D printing or additive manufacturing to encapsulate the bacteria within the material and by electrospinning. These materials can be applied for cell engineering, as biosensors, as skin patches for wound healing and for controlled drug release.

Recognized as Safe (GRAS) species of bacteria, yeast and unicellular algae, as has been demonstrated with living materials containing *E. coli* Nissle 1917 (REF.[26]), *Lactococcus lactis*[23,32], *Saccharomyces cerevisiae*[33] and *Spirulina*[34].

The integration of functional microorganisms into polymeric matrices imposes stringent requirements on materials engineering. The matrix has to support survival and metabolic function of the encapsulated organisms, and confine them to the application site. In this Review, we discuss the parametric window of material precursors and processing conditions that are compatible with living components, and highlight the advantages and disadvantages of different microorganisms and polymeric matrices (TABLES 1,2).

Traditional living materials

Living material designs have long been used for probiotics and biocatalysis. Here, microorganisms are encapsulated in polymeric matrices, typically in the form of microcapsules or fibre meshes, to facilitate the delivery of the microorganism to the application site or to isolate the microorganism after reaction.

Probiotics. Probiotics are microorganisms expected to confer health benefits[35–37]. Probiotics are also considered advantageous for food packaging, because bacteria can decrease the oxygen permeability of the packaging material, which is beneficial for food preservation[38,39]. Probiotic treatments require the ingestion of about 10^9 viable cells per day[40]. To preserve viability, probiotics can be encapsulated in hydrocolloids, lipids and polymer composites[41–43], which protect them against low pH and bile present in the gastrointestinal tract[40]. Hydrocolloids, including polysaccharides, such as cellulose and derivatives, chitosan and alginate[44,45], proteins, such as gelatin, wheat gluten, corn zein, collagen, soy protein and casein[46], and lipids, such as natural waxes and acylglycerols[46], are biodegradable and can release the probiotic strains after passage through the gastrointestinal tract. Food products, such as yogurts, frozen dairies and cheese, can include probiotics without compromising their organoleptic characteristics. The addition of probiotics leads to noticeable effects on the gut microflora, suggesting a successful transit through the gastrointestinal tract and in situ probiotic cell release[47]. For example, encapsulation of *Lactobacillus* in alginate and soy protein isolate significantly increases its survival in a simulated gastrointestinal environment, compared with free cells[35]. However, safety concerns have been raised regarding probiotics potentially altering the microflora ecosystem, which need to be further assessed[47].

The next frontier in probiotic therapies involves their genetic modification to provide them with sensors and switches for programmed therapeutic functions[48]. In particular, common probiotic lactic acid bacteria have been genetically modified and are currently in clinical trials. For example, *L. lactis* has been engineered to secrete anti-inflammatory drugs to combat inflammatory bowel disease[49,50] and *Lactobacillus jensenii* has been engineered to produce antivirals to inhibit vaginal HIV entry[51]. These bacteria are undergoing clinical trials (Precigen Actobio[52] and Osel Inc.[53]) and several others are in development[54]. The delivery and sustenance of such bacteria are expected to be further improved by encapsulation in materials.

Biocatalysts. Progress in biocatalysis has contributed considerably to our understanding of bacteria–material interactions. Biocatalysis refers to reactions catalysed by enzymes immobilized on a solid substrate or by entire cells in a reaction medium. Here, we only refer to the latter. Bacteria have long been used as biocatalysts, with a first report in 1911, describing furfural reduction by yeast during alcoholic fermentation, and a publication in 1948, reporting microbiological oxidation of sterols[55,56]. Notable examples of whole-cell biocatalysts include polyelectrolyte microparticles containing a recombinant strain of *E. coli* that expresses cyclopentanone monooxygenase to catalyse Baeyer–Villiger biooxidations, *Corynebacterium glutamicum* for the production of L-lysine and L-glutamate[57], *Paracoccus denitrificans* for vitamin B_{12} biosynthesis[58] and *Agrobacterium*/*Rhizobium* HK1349 for industrial-scale biosynthesis of L-carnitine[59,60].

Engineered living biofilms can also be used in bioreactors for biotechnological production. The high cell density within biofilms and the ability of biofilms to colonize substrates make them ideal materials for a retentostat[61]. In such a chemostat (bioreactor), the catalyst, which are whole cells in this case, are retained within the bioreactor for the production of high-end biotechnological products, for example, cephalosporin C,

amylase and cellulase[62]. However, the reactions catalysed by intracellular enzymes often compete for cofactors with the microorganism metabolism, which can negatively affect the productivity and survival of the cells. Thus, cascade catalysis has been explored to improve whole-cell biocatalysis by physically confining the required enzymes in organelles (peroxisomes, mitochondria and capsosomes)[63]. Locating enzymes of the same pathway in close proximity increases the yield and reduces the metabolic burden of the host cell. Alternatively, enzymes can be displayed in microbial membranes to facilitate access to the substrates, avoiding the bottleneck of transmembrane transport of the molecules involved in the catalysed reactions[64].

Biomedical applications of living materials

Living materials have been developed and optimized for biomedical applications, including biosensors, skin patches for wound healing, drug delivery systems and tissue engineering constructs.

Encapsulated whole-cell biosensors. Gene expression[65] in microorganisms can be engineered to be activated in response to medically relevant molecules, such as glucose, maltose, oestrogens and quorum-sensing molecules of pathogens, for the development of whole-cell microbial biosensors[66,67]. Bacterial biosensing can be realized by modifying kinases, transcription factors, repressors or RNA riboswitches, which can then activate the production of a reporter protein in response to the binding of an analyte[66]. In general, reporter signals are fluorescent or bioluminescent proteins, which allow easily quantifiable optical readouts. Such a sensing and reporting mechanism enables signal amplification, because a single analyte molecule can induce the production of multiple copies of the reporter protein[68]. Furthermore, the production of whole-cell living biosensors is cost-effective, because, once the sensing circuits have been encoded, the bacteria just need to be grown and encapsulated in matrices[66,69,70].

Such a living biosensor can, for example, detect isopropyl-β-D-1-thiogalactopyranoside (IPTG), lactose or galactose in milk by encapsulating genetically engineered *E. coli* in agar hydrogels, sandwiched between polystyrene and nanoporous polycarbonate membranes[71]. Using this biosensor, analytes can be detected in very low volumes (1–10 µl) of milk within 30–120 min. Owing to the poor mechanical stability of agarose and the requirement for cold storage, these biosensors need to be produced in a batch process, limiting them to laboratory-scale production and a cost of 2.50 USD per sensor unit[72]. Alternatively, spore-forming *Bacillus subtilis* can be encapsulated in polyvinyl alcohol (PVA) hydrogels, which can be dried, allowing biosensor

Table 1 | **Microbial strains in living materials**

Strain	System	Advantages	Disadvantages	Refs
Escherichia coli Nissle 1917	Biosensor pill for gut bleeding; IBD treatment	Probiotic strain for the GI tract; easy to modify	Endotoxins	26,70
Escherichia coli ClearColi	Light-responsive drug delivery	Endotoxin-free strain; easy to modify	Not a probiotic	29,30
Escherichia coli lab strains	Biosensors; living tattoo; biocatalysis	Fast growth; large genetic toolbox	Endotoxins	14,69,71, 105,186
Lactococcus lactis MG1363/NZ9000	Living biointerfaces	Probiotic strain; adhesive biofilms; good at protein display and secretion; endotoxin-free strain	Limited genetic toolbox	23,90–92, 125
Bacillus subtilis	Low-cost biosensors; skin patches	Forms robust spores; antifungal properties; Gram-positive model organism	Moderate genetic toolbox	24,25,72
Staphylococcus epidermidis	Skin patches	Skin commensal; antifungal properties	Opportunistic pathogen inside tissues	79
Lactobacillus; *Bifidobacterium*; *Streptococcus*; *Enterococcus*	Probiotic microcapsules	Probiotic strains for the GI tract	Limited genetic toolbox	41–46
Acetobacter xylinum	Living skin graft	Bacterial cellulose producer	Not a probiotic; not genetically tractable	138
Spirulina	Stem cell, neuroregenerative and anti-thrombogenic scaffold	Anti-inflammatory; antibacterial; antifungal; antimetastatic; antioxidant; regenerative	Only lysed extracts used for these applications	135–137
Penicillium chrysogenum	Drug release	Naturally produces penicillin	Minimal availability of genetic tools	28
Saccharomyces cerevisiae	Biosensor; biocatalysis; bioremediation	Food-grade; cheap; large genetic toolbox	Ethanol and CO_2 production undesirable for some biomedical applications	73,132, 150,151

GI, gastrointestinal.

Table 2 | **Materials used in living materials**

Material	System	Advantages	Disadvantages	Refs
Agarose	Drug release; 3D printing	Good for sustaining bacterial populations; gelation by cooling to room temperature	Uncontrolled bacterial growth	28–30
Pluronic F-127	Biosensors; skin patch; 3D printing	Gelation by heating to room temperature; chemically modifiable; physical and chemical crosslinking possible; shear thinning	The physically crosslinked gel dissolves in water	14,25, 31
PVA	Electrospinning; cheap biosensor	Forms porous matrix during drying	Water-soluble	72,127, 129
Alginate	Cheap 3D printing, probiotics	Widely used; forms gel in contact with Ca^{2+}; good printability; chemically modifiable; cheap; accessible	Needs Ca^{2+} to remain a gel; physical crosslinking only	125
PEO	Electrospinning; tissue engineering scaffolds	Water-soluble; substitutive for organic solvents in electrospinning	Can increase water solubility in polymer blends	135
PCL		Thermoplastic; low melting temperature; medical-grade PCL available	Not suitable for live-cell encapsulation during electrospinning	136
Silk fibroin		Genetically modifiable polymer	Expensive	137
Curli nanofibres[a]	Engineered biofilms from *Escherichia coli*	Extensively genetically modifiable	Biodegradable; relatively soft	7,26,85

PCL, polycaprolactone; PEO, polyethylene oxide; PVA, polyvinyl alcohol. [a]Produced by bacteria in situ, followed by extracellular self-assembly.

production by an industry-standard, roll-to-roll coating process[72] (FIG. 2a), at a cost of 0.0009 USD per disc. The dormant spores in these hydrogels can be stored long term (at least 4 weeks) at temperatures between −20 °C and 80 °C, and revived with medium to yield functional biosensors. These bacteria were programmed to sense IPTG and produce a fluorescent signal that could be observed within 1 h of exposure to IPTG concentrations as low as 1 mM.

Wearable biosensors can detect biochemicals in body fluids (for example, in sweat), enabling non-invasive, real-time health monitoring. Living biosensor devices can be made of flexible materials for sensitive, multiplexed analyte detection with improved wearability compared with purely hydrogel-based living materials[14,69]. For example, a sensor can be made of elastomeric compartments, which support gas exchange, and contain bacterial cultures that are sealed off by tough polyacrylamide–alginate hydrogels, allowing diffusion of nutrients and analytes[69]. This device is stretchable up to 1.8 times its original length and capable of detecting different small molecules (IPTG, rhamnose and *N*-acyl homoserine lactone (AHL)) when applied on skin or nitrile gloves[69]. The bacteria can also be printed using Pluronic F-127 diacrylate as ink to engineer a flexible living tattoo[14], which can sense IPTG, rhamnose and AHL on skin (FIG. 2b). Similarly, agarose-based biosensors encapsulating *B. subtilis* spores[24] can survive a temperature of 72 °C, which is required for 3D printing of this living material. In the presence of suitable nutrients and oxygen, the spores can germinate and are genetically programmed to report the presence of IPTG, xylose, vanillic acid or cuminic acid by producing green fluorescent protein (GFP) (detectable in about 5 h from application of analyte)[24].

In addition to external biosensing, living biosensors can also be designed to sense within the body. For example, an ingestible bacterial electronic capsule can sense gastrointestinal bleeding[70] (FIG. 2c). Here, *E. coli* was engineered to detect haem at titres as low as 0.2 µM or 10 ppm and produce bioluminescent luciferase. The bacteria are encased in an acrylonitrile butadiene styrene (ABS) plastic chamber, which is closed off on one side by a transparent polycarbonate window and on the other side by a semipermeable membrane to allow analyte diffusion. These bacterial containers are then encased in polydimethylsiloxane (PDMS), together with a miniature photosensing electronic device, forming an ingestible capsule that can detect internal bleeding within pig guts and wirelessly relay the information in real time. A self-grown living material biosensor based on co-culturing of engineered yeast cells and bacterial cellulose-producing *Komagataeibacter rhaeticus* bacteria[73] can detect the oestrogen steroid hormone β-oestradiol through genetic engineering of the yeast. β-Oestradiol is an environmental pollutant affecting aquatic species and humans. The sensor can detect titres as low as 5 nM and produce a fluorescent protein in response.

Synthetic biology advances have opened the doors to a wide range of possibilities for living biosensors. Furthermore, microtechnologies and nanotechnologies have been developed that enable easy handling, portability and multiplexing of sensing[74]. Genetic circuit designs further allow the simultaneous sensing of multiple analytes, execution of logic functions and tunability of sensitivity ranges of detection[75,76].

Skin patches restore the skin microbiome. The skin hosts a rich microbiome with multiple bacterial and fungal species coexisting in symbiosis, and is also available to topical application of living materials. The dominance or recession of certain species can lead to dysbiosis that manifests as skin disorders, such as acne vulgaris, atopic dermatitis and psoriasis[77]. Microbial therapies for the treatment of such disorders are being explored; however, introducing therapeutic microorganisms to the skin may raise the risk of dysbiosis, if the microorganisms disproportionally colonize the treatment site[78]. To alleviate this risk, therapeutic skin-commensal bacteria can be entrapped within stabilizing matrices that control the outgrowth of bacteria that colonize the affected skin region. For example, protective skin dressings can be designed by encapsulating *Staphylococcus epidermidis* in polyelectrolyte-based nanofibrillar mats[79]. These mats limit bacterial metabolism and delay bacterial outgrowth by ~5 h in shaking culture conditions in vitro. Bacterial metabolism can be slowed down 2–3-fold, compared with free bacteria, and outgrowth rates can be delayed to ~6 h by varying the number of polyelectrolyte layers.

Similarly, antifungal living skin patches can be designed by incorporating the wild type *B. subtilis* 3610 strain, which naturally produces the antifungal compounds surfactin, fengycin and iturin[25], in a thermo-responsive gel made of the triblock copolymer Pluronic F-127 (REF.[25]). A solution of this polymer remains fluid at 4 °C but forms a viscous gel above 15 °C, allowing easy bacterial incorporation and application on the skin. By varying the polymeric and bacterial compositions, the mechanical properties of the gel can be tuned, for example, to ensure penetration of the gel precursor solution into the epidermal layer but not into the dermal layer, prior to gelation. In a mouse model, this skin patch could limit a *Candida albicans* infection, which is one of the most frequent causes of skin infections. However, whether the gel disperses and whether *B. subtilis* is incorporated in the skin microbiome remains to be explored. In these applications, living materials ensure the controlled release of the entrapped bacteria to re-establish the symbiotic coexistence of microorganisms in the skin.

Self-growing biofilms as wound patches and tissue adhesives. Biofilm engineering for biocatalysis[80], environmental remediation[81], underwater adhesion[82], gradient mineralization[83] and other applications[7] has been widely performed using *E. coli* programmed to secrete curli nanofibres. Curli nanofibres are major biofilm matrix proteins involved in bacterial surface adhesion, aggregation and induction of host inflammatory responses[84]. The 13-kDa curli subunit CsgA is secreted out of the bacterial cell, where it assembles into amyloid fibres with the help of bacterial-surface-associated CsgB, which induces its nucleation[7]. This efficient self-assembly process leads to the formation of mechanically stable matrices made of fibres, which, in combination with their genetic tractability, have made CsgA an ideal candidate for the development of self-regenerating functional living materials[7].

Most *E. coli* strains used in these living materials do not produce curli fibres on their own, and, thus, only recombinantly modified and encoded curli fibres are present in their matrix[7]. For example, for the treatment of inflammatory bowel disease[26], the *E. coli* strain Nissle 1917 was programmed to secrete CsgA curli fibre proteins fused to trefoil factors (TFFs), which are known to promote intestinal barrier function and epithelial restitution. In a mouse model, in which colitis is induced by dextran sodium sulfate, these biofilms can be orally administered and adhere to intestinal ulcers, where they form a protective biointerface that promotes mucosal healing and immunomodulation[26] (FIG. 3a). Similar biofilms have been engineered to selectively capture virus particles from water[85] by fusing the CsgA protein to the influenza-virus-binding peptide C5. The biofilms can be attached to polypropylene filler materials to construct purification columns for the disinfection of river water, with influenza titres as high as 10^7 PFU l^{-1}. A genetic kill switch limits the growth of the biofilm by inducing bacterial lysis once a threshold population density is reached. The bacteria can sense whether their own quorum-sensing molecules exceed a certain threshold concentration and, in response, produce a lysis protein (ϕx174E), which kills them. Therefore, bacterial population growth is limited to a density that allows effective capture of virus particles[85]. This curli-based system can also be tailored to repair damaged bleeding vascular tissues[27] by engineering the bacteria to sense haem in the environment and produce adhesive curli fibres in the form of a glue, which seals leaky membranes (FIG. 3b). In an in vitro microfluidic device that contains flowing horse blood and a semipermeable membrane, from which blood can leak, the bacteria can detect the leakage and completely seal the membrane.

Other types of bacteria, such as *B. subtilis*, have been engineered based on the TasA amyloid machinery of *B. subtilis*, whose properties can be tailored to have the adequate viscosity to be 3D printed and display self-regeneration properties[86].

Living instructive scaffolds support tissue regeneration. Research on materials for biomedical implants has shifted towards the development of responsive materials that can instruct embedded cells to support tissue development and maintenance[87,88]. Materials containing microorganisms can be engineered to react to and remodel the cellular microenvironment[89], in response to physical stimuli, for example, temperature, light or other electromagnetic fields, and mechanical forces, or chemical stimuli, for example, pH, chemical species or of concentration gradients[5].

For example, *L. lactis* biofilms can be genetically engineered to produce extracellular matrix proteins and growth factors in situ for tissue engineering applications[23,90–92] (FIG. 3c). *Lactococcus lactis* MG1363,

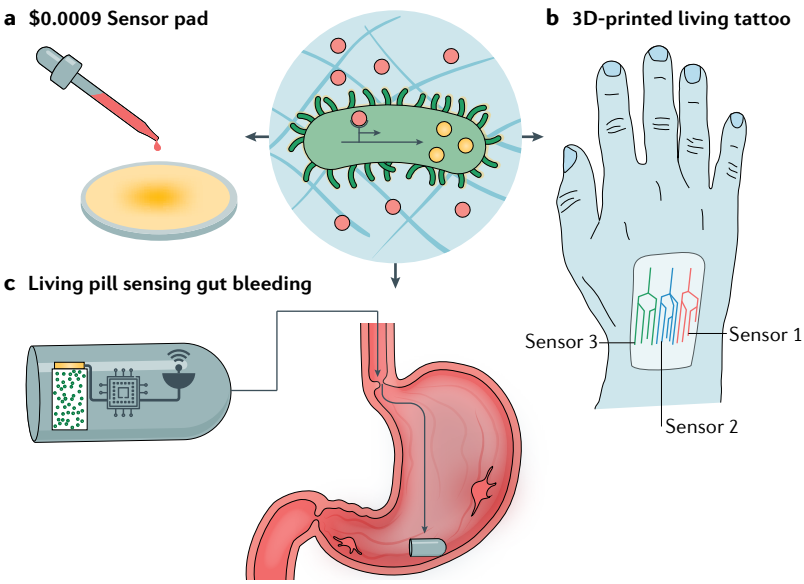

Fig. 2 | **Living materials as biosensors.** Engineered bacteria can be encapsulated in a matrix to detect and respond to analytes by generating a fluorescent signal. **a** | Simple biosensors can be mass-produced at low cost by embedding engineered microorganisms in a paper substrate to detect analytes or contaminants[72]. **b** | A tattoo sensing contamination of the skin can be used for the detection of small molecules[69]. **c** | A gut-bleeding-sensing pill wirelessly transmits the results of the detection[70] and can be mass-fabricated at low cost.

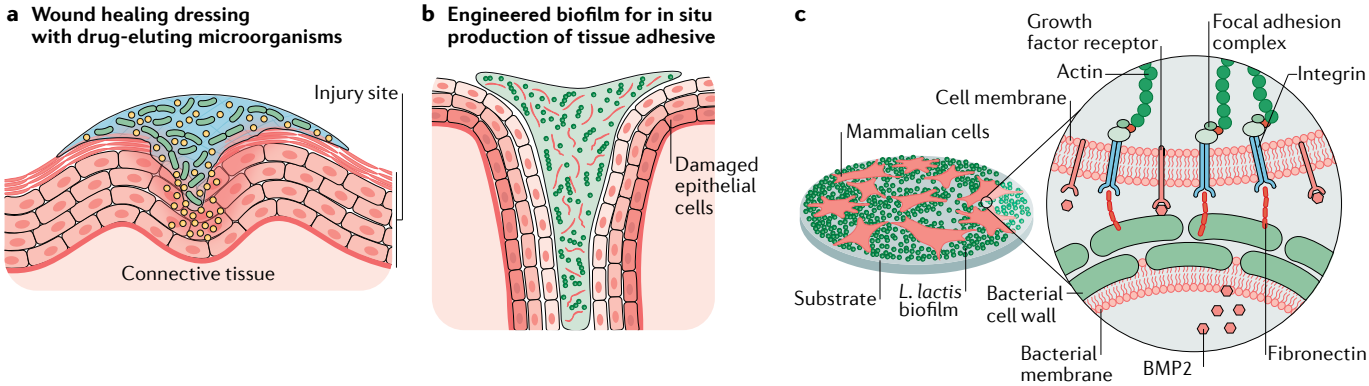

Fig. 3 | **Living materials as tissue adhesives. a** | Biomaterials with engineered microorganisms can produce and elute recombinant proteins or small molecules in situ to aid wound healing in chronic wounds (such as burns and diabetic wounds); for example, the *Escherichia coli* strain Nissle 1917 produces recombinant CsgA curli fibres fused to trefoil factors to restore epithelial function. **b** | Engineered bacteria in living materials can be engineered to produce tissue adhesives, that is, curli fibres, to help in the recovery of wounded tissues. **c** | The biofilm-forming ability of *Lactococcus lactis* can be exploited for the engineering of biofilms, which can act as an interface between a biologically inert substrate and mammalian cells. *L. lactis* produces different biologically active proteins that trigger the adhesion and differentiation of mammalian cells cultured on top of the biofilm. BMP2, bone morphogenetic protein 2.

a plasmid-free strain, and its derivative *L. lactis* NZ9000, which has an engineered nisin-controlled gene expression system (NICE)[93], can be designed to express proteins involved in cell adhesion, proliferation and differentiation, such as fragments of fibronectin, which is an important extracellular matrix protein[94]. The fibronectin fragment FN III_{7-10} harbours an arginine–glycine–aspartic acid (RGD) integrin-binding motif in the ninth type III repeat and a PHSRN or synergy motif in the tenth type III repeat. *L. lactis* can be engineered to express the FN III_{7-10} fragment modified with a secretion peptide at the N terminus, which is cleaved upon secretion, and a *S. aureus* SpA cell-wall binding motif, allowing the fragment to be secreted, followed by covalent binding to the cell wall. The lack of lipopolysaccharides in the cell wall of *L. lactis* enables direct interaction of RGD-ligating integrins on mammalian cells with the fibronectin fragment. Therefore, a biofilm of *L. lactis* constitutively expressing FN III_{7-10} interacts with mammalian cells through integrin–fibronectin adhesion; thus, the bacteria become the growth substrate for mammalian cells. Such *L. lactis* biofilms can be applied in bioreactors to control stem cell fate and could also be used in vivo, if adequately encapsulated. The ability of *L. lactis* to produce different proteins and certain small molecules on demand allows control of the differentiation process without the need for external biochemical factors.

E. coli has also been applied for the design of light-responsive living biomaterials[95]. Most laboratory *E. coli* strains do not develop biofilms and contain immunogenic lipopolysaccharides on the outer membrane. However, the *E. coli* strain ClearColi BL21(DE3) does not produce endotoxic lipopolysaccharides and can be electrostatically immobilized on glass surfaces coated with positively charged poly-L-lysine, owing to its negatively charged outer membrane. Irradiation with light causes cleavage of the photosensitive IPTG, which then induces the expression of a cell-adhesion-promoting RGD knottin[96] miniprotein on the bacterial surface (FIG. 4a). This is the first example of a light-responsive living biomaterial, in which an optogenetic stimulus, such as blue light, can be used to induce the production of a protein that enables the interaction of the bacteria with mammalian cells, in this case, through RGD–integrin interactions[95].

Self-replenishable and personalized drug-eluting materials. Microorganisms are commonly used for the production of biopharmaceuticals (for example, peptides, proteins and oligonucleotides), which are drugs derived from natural sources with a range of therapeutic functions, from infection treatment to gene therapy[97]. In addition to engineering microorganisms for drug production, they can also be programmed as drug delivery vehicles. These live biotherapeutics[98] can site-specifically produce and deliver drugs in situ[48,54,78]. A number of live biotherapeutics are currently in clinical trials for the treatment of disorders in the gastrointestinal and genitourinary tracts, skin and oral cavity[48,54,78]. Live biotherapeutics enable a personalized medicine approach, because microorganisms can be programmed with sensors and switches to deliver the drug in response to specific disease markers. However, potential uncontrolled, opportunistic microbial growth and colonization may damage sensitive tissues[99,100]. The colonization behaviour of newly introduced engineered bacteria depends on the patient, organ and medical condition, and is, thus, difficult to standardize, as is often the case with probiotic therapies[101] in the gut. Consequently, predicting or controlling drug profiles, which is important in therapies requiring tight regulation of drug doses (for example, growth factors, insulin, cytokines), remains challenging[48,54,78].

To overcome these drawbacks and expand the applicability of live biotherapeutics, living drug-eluting implants that encapsulate microorganisms in polymeric matrices have been explored[24,28–31]. The polymeric

matrices provide a supportive environment for the survival of a microbial population and can be designed to ensure that the microorganisms do not escape. Adequate porosity in the polymeric matrix allows diffusion of nutrients, gases, metabolites and drugs in and out of the material. Although not yet tested in vivo, the nutrients and gases required for the sustenance of microorganisms in living drug-eluting implants are expected to be available in the body, similar to how the microbiome is sustained. In contrast to non-living drug-release devices, the drugs in drug-eluting living materials can be produced in situ and long-term controlled release is theoretically possible. Therefore, this strategy is expected to be more cost-effective compared with traditional drug delivery systems, which require drug purification, storage and packing steps. Drugs can be freshly produced in real time and, therefore, living therapeutic materials are ideal for the delivery of fragile protein-based drugs. Moreover, smart functions can be introduced without increasing the complexity of the material by genetically encoding the microorganisms with sensors and switches. These advantages are propelling the development of live biotherapeutics in the form of living materials.

One of the first examples of a drug-eluting living material for sustained antibiotic release was fabricated by encapsulating penicillin-producing fungi in an agar-based hydrogel, sandwiched between a polyacrylate layer and a nanoporous (400 nm pore diameter) polycarbonate membrane[28]. This construct releases the antibiotic for at least 10 days, killing Gram-positive bacteria, such as *S. carnosus*. However, in this system, the bacteria were not genetically programmed to provide control over release. To control drug release, we fabricated agarose-based hydrogels encapsulating optogenetically engineered *E. coli*, which allows light-regulated release of a red fluorescent protein (RFP)[29] and the antimicrobial and antitumoural drug deoxyviolacein[30] (FIG. 4b). Here, the endotoxin-free *E. coli*, ClearColi, was programmed using an optogenetic module that activates drug production in response to blue light irradiation. Controlled and localized drug production could then be remotely triggered by modulating the exposure and spatial confinement of incident light (FIG. 4c). In transwell inserts with a nanoporous membrane interface, the drug could be released into external medium without bacterial escape. Dosage of release could further be modulated

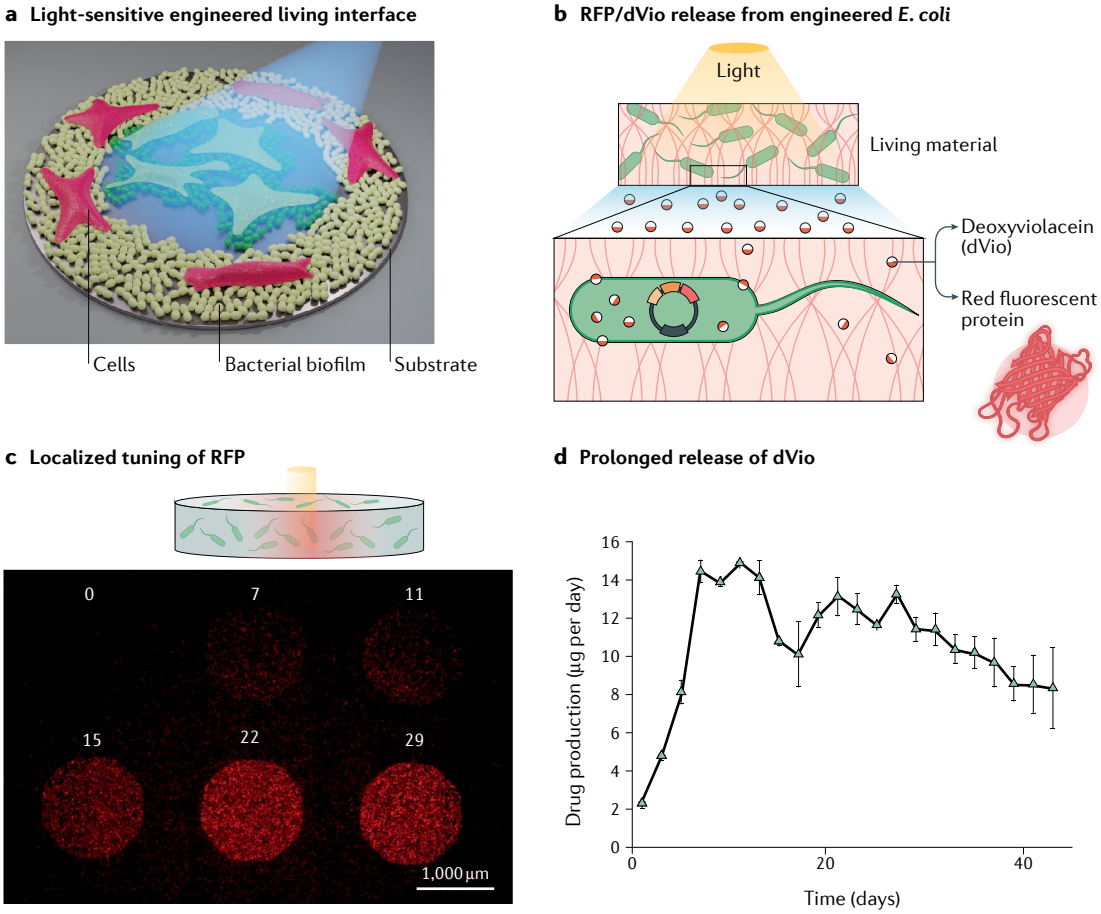

Fig. 4 | **Light-responsive living therapeutic materials. a** | Living interfaces can be engineered to display cell-adhesive ligands and promote intracellular drug delivery in response to light, in a spatially controlled manner[95]. **b** | *Escherichia coli* can be optogenetically engineered to produce and release either a protein (red fluorescent protein (RFP))[29] or the small-molecule drug (deoxyviolacein (dVio))[30]. **c** | The production of molecules within living materials can be locally triggered and tuned by varying the duration and intensity of light irradiation. **d** | Drug production can be sustained for more than a month. Part **c** reprinted with permission from REF.[29], Wiley. Part **d** adapted with permission from REF.[30], Wiley.

by varying light exposure, enabling prolonged release for over a month[29,30] (FIG. 4d). These proof-of-concept studies demonstrate the potential of controllable drug-releasing living materials, which can then be adapted for specific therapies.

Pluronic F-127 bisurethane methacrylate has also been explored as a chemically crosslinked bioink for the encapsulation and 3D printing of bacteria engineered to secrete the peptide antibiotic colicin V[31]. These living materials can be fabricated in desired geometries, stored and reused multiple times through freeze drying, and they can inhibit the growth of Gram-negative bacteria, as shown by zone of inhibition assays on agar plates[31]. However, potential outgrowth of bacteria into the external medium and remote control of antibiotic production remain to be addressed. 3D-printed agarose–*B. subtilis* systems have also been engineered for antimicrobial drug release[24], enabling controllable drug release; however, bacterial outgrowth into the external environment would also need to be assessed. Here, *B. subtilis* was engineered to release the antimicrobial lysostaphin or thiocilline in response to vanillic acid or IPTG, respectively. These living materials can be directly printed onto and seal wounds, preventing *S. aureus* growth, as tested by a growth-inhibition assay on agar plates[24]. Similarly, *E. coli* can be engineered to autonomously detect and kill *S. aureus*. The bacteria can be encapsulated within a matrix, to which they adhere, which limits their outgrowth from the material[102]. These *E. coli* were engineered to display an adhesion protein, allowing them to bind glucose and establish strong interactions with dextran-based gels with large pore diameters (10–100 μm). Outgrowth into the surrounding medium could not be completely prevented but reduced by 100-fold compared with encapsulated *E. coli* without the adhesive capability. This living material can inhibit the growth of methicillin-resistant *S. aureus*.

These studies highlight the versatility of living materials in terms of bacterial engineering and materials processing. However, current systems often require a few hours to a few days to achieve therapeutically relevant doses. The rate of drug production and release, therefore, remains to be improved, for example, by fine-tuning of genetic circuits and by optimizing hydrogel porosity and diffusivity. In addition, biocompatibility and biosafety need to be tested in suitable animal models to ensure compatibility of both bacterial and material components, in particular, in terms of immune responses.

Microorganism encapsulation

Cells can be physically embedded in polymeric matrices by whole-cell trapping or encapsulation[103,104]. For example, cells can be entrapped within a rigid network, which prevents them from diffusing into the surrounding medium and allows penetration of the substrate[105]. Ionic hydrogels, thermogels and synthetic or natural polymers are commonly used as substrates. To achieve immobilization in ionic hydrogels, alginic acid[46] and κ-carrageenan are often used, because these materials can be crosslinked by divalent cations, such as Ca^{2+} or Mg^{2+}. For example, bacterial strains expressing bioluminescent enzymes can be encapsulated in alginate hydrogels for water sensing applications[106]. Natural polysaccharides, such as agar or agarose, are used as thermogels for encapsulation. The sol–gel transition temperature of these materials is in the range of 32–43 °C; however, the main limiting factor is the transition temperature, because it can compromise the viability of the encapsulated cells. Alternatively, PVA, polyacrylamide and sol–gel matrices are more stable than natural polysaccharides, owing to their orthogonality to biodegradation processes[107]. Cells can also be encapsulated in polyelectrolyte complex capsules[60,108]; here, water-soluble polyanions and polycations interact to from microparticles with diameters of ~1 mm (REF.[60]). These microparticles have a viscous core formed by complexation of the polyelectrolyte and the cells, and, optionally, an outer shell[60,108].

Biofilm engineering. Biofilms are a prime example of a natural living material. In a biofilm, sessile microbial consortia are embedded in a 3D matrix composed, at least partially, of material produced by the microbial community[109]. To promote biofilm formation, bacteria can be passively or actively immobilized on a surface. For example, coatings with adhesive molecules, such as poly-L-lysine[110] or SpyTag/SpyCatcher[111], can promote the attachment of cells to the surface and to each other. Passive immobilization relies on the physical adsorption of bacteria to natural substrates, leading to colonization and biofilm formation[112]. Biofilm applications have the advantage that bacteria can self-immobilize onto a substrate without the need for chemical or physical crosslinkers, making the biofilm stable in its physiological state. Thus, genes of interest can be cloned under the control of promoters that are upregulated in sessile physiological states. However, bacterial proliferation is difficult to control in biofilms. Cells eventually leave the biofilm into the planktonic phase, and the intrinsically limited life cycle of the biofilm further limits its application time[113].

Several factors influence bacterial adsorption onto a surface; most bacterial strains have negatively charged membranes and, therefore, a positively charged surface promotes the first stages of biofilm development, in which planktonic cells adhere to surfaces. Topography also plays an important role in bacterial adhesion. Bacteria colonize and proliferate better on micropatterned surfaces than on flat substrates, owing to the development of more stable biofilms[114,115]. The bacterial species is also a key factor. Some bacteria develop thick and stable biofilms, whereas some bacteria develop only monolayered, weak biofilms. Substrates adequate for biofilm support should have four main characteristics; they should favour adhesion of the microorganisms to the substrate, have adequate mechanical resistance to stresses exerted on them, be manufacturable (and affordable) and have broad availability. In addition, materials should have adequate porosity, microtopography (roughness), net charge, surface energy or hydrophobicity, and they can be inorganic or organic[11,114–119].

Microencapsulation. Microencapsulation of microorganisms has been widely used for the storage and delivery of probiotics[120,121]. Microencapsulation protects

bacteria from the harsh acidic environment of the stomach, allowing viable transit through the gastric juices, enabling eventual colonization of the bacteria in the intestine. Polymeric matrices for encapsulation should be acid-tolerant and biodegradable, and, ideally, cost-effective, with good palatability. Various food-grade polymers fulfil these requirements and, thus, may be explored for bacterial microencapsulation in biomedical applications[120,121], for example, alginate, κ-carrageenan, gellan gum, xanthan gum, starch, chitosan, gelatin and casein. Common techniques for microencapsulation include spray drying, extrusion-based droplet generation and emulsion formation. Each technique has its own advantages and disadvantages, and the selection of the encapsulation technique depends on the probiotic, polymer and the properties necessary for the desired living material application. For example, spray drying and emulsion provide high throughput at low cost, although variations in size are typically greater than in extrusion techniques, which can provide well-defined and monodisperse particles. These techniques can also be applied to make core–shell hydrogels, wherein bacteria are entrapped within an inner microgel covered by an enveloping gel, which provides additional or complementary protection against external factors (such as pH, temperature and chemicals)[120,121]. Microfluidic devices can also be used to generate cell-laden monodisperse droplets; here, size, geometry and composition can be precisely tuned[122].

These technologies have been widely used for the creation of microgels containing probiotics[103,120,121] and mammalian cells[122,123]; however, few studies have reported their use for microbial encapsulation for biomedical applications. For example, bacteria engineered to detect a *Pseudomonas aeruginosa* quorum-sensing molecule (*N*-(3-oxododecanoyl)homoserine lactone) and producing a reporter fluorescent protein can be encapsulated in alginate methacrylate microgels (100–200 μm diameter) by electrostatic extrusion in a calcium chloride bath to ionically crosslink them[124]. These gels can be further crosslinked by ultraviolet light to photoinitiate radical polymerization without affecting bacterial viability. Although bacteria can leach out of these microgels, the covalent crosslinking reduces leaching by ~10-fold compared with gels that are not covalently crosslinked. This living material can be used for biosensing, with a detection limit as low as 0.1 μM, allowing the detection of a *P. aeruginosa* infection[124]. These microgels can be further embedded in polyethylene glycol (PEG) hydrogels to form a dipstick that can detect the presence of *P. aeruginosa*. Living microgels can also be made of alginate for tissue regeneration. Using extrusion, alginate microgels can be engineered as defined 3D environments for the encapsulation of bacteria and mesenchymal stem cells. The bacteria (*L. lactis*) are engineered to express BMP2, which directs osteogenic differentiation of the stem cells. Thus, each microgel can serve as an independent microenvironment for drug testing[125].

Spinning. Spinning is a process in which a viscous polymer fluid is drawn into fibres with diameters at the submicron to millimetre scale. There are multiple spinning techniques, including dry, melt, wet and electrospinning, of which only the latter two have been used for the fabrication of living materials, most likely owing to the fact that dry and melt spinning require harsh conditions that would affect microbial survival[126–128]. In wet spinning, a polymer solution, which is first made in a particular solvent, is extruded into a coagulation bath, which contains a different solvent that functions to precipitate the polymer solution into solid fibres. This technique has been applied to encapsulate bacteria, such as *Micrococcus luteus*, *Nitrobacter winogradskyi* or *Shewanella oneidensis*, in PVA fibre meshes[127,129,130]. Aqueous PVA solutions, made at 120 °C under pressure, are first cooled to enable homogeneous mixing with the bacteria, followed by extrusion in acetone. The resulting 50–200-μm-thick fibres can then be manually collected on a metal frame. These PVA fibres are soluble in water and can, thus, be stabilized by chemical vapour deposition of a thin-layer coating (~5 μm thickness) of hydrophobic poly(p-xylylene) (PPX)[127,129] (FIG. 5a). These harsh processing steps result in a layer of dead bacteria near the outer surface of the fibres; however, bacteria in the core of the fibres survive and proliferate throughout the fibres in the presence of growth medium[127,129]. Alternatively, an automated device can be used to pull the fibres from the acetone bath at precisely controlled rates[130]. Long exposure times to acetone (25 s) increases the crystallinity of the PVA fibres (43%), compared with manual drawing (33%), resulting in stable PVA fibres that do not dissolve in water over several days. Long acetone exposure also increases the thickness of the shell of dead cells near the surface of the fibres; however, surprisingly, after culture in medium, the remaining live cells do not grow into the region of dead cells, possibly indicating a different, more stable, molecular structure of PVA near the surface. Therefore, this strategy allows wet spinning of mechanically stable living biocomposite fibres that do not require any further post-processing.

In electrospinning, jets of electrically charged polymers are manipulated by strong magnetic or electric fields to draw fibres with submicron diameters. Although this technique is seemingly harsh, involving high voltages in the range of several kilovolts, it has been widely used to incorporate living organisms in fibre meshes[126]. A variety of polymers, including polycaprolactone (PCL), pluronic derivatives, PVA, polyvinylpyrrolidone (PVP), PEG and silk fibroin, have been used to encapsulate bacteria, yeast, algae and viruses[126]. Encapsulation has been achieved in single-material fibres, core–shell fibres and microgel-in-fibre formats. For single-material fibres, cytocompatible and water-soluble polymers are required that can be mechanically stabilized during or after spinning. For example, aqueous Pluronic F-127 dimethacrylate solutions, which are liquid at 4 °C, form non-covalently stabilized gels above room temperature and can be covalently crosslinked using free-radical generators[131]. Accordingly, bacteria (*Zymomonas*, *E. coli*) can be encapsulated in electrospun fibres by homogeneous mixing in a polymer solution at 4 °C, followed by spinning at room temperature and chemical crosslinking using ammonium persulfate and ascorbic acid in a glycerol bath. Here, glycerol is required as organic solvent

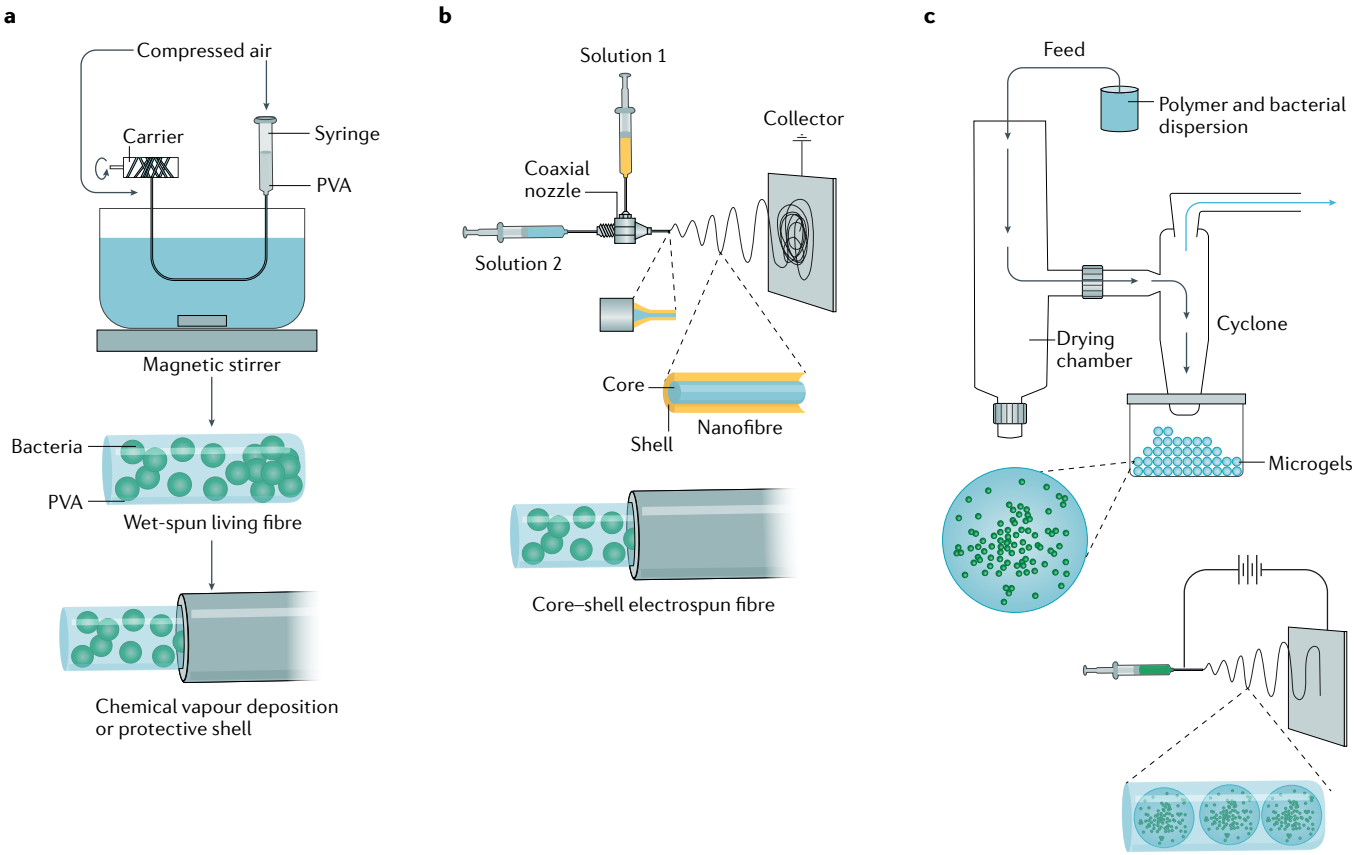

Fig. 5 | **Spinning techniques for the fabrication of living materials. a** | Wet spinning of bacteria in polyvinyl alcohol (PVA) followed by chemical vapour deposition of a hydrophobic poly(p-xylylene) (PPX) shell, which prevents dissolution of PVA and bacterial escape[127,129]. **b** | Electrospinning of core–shell fungal fibres made of a polyvinylpyrrolidone (PVP) core and a polyvinylidenefluoride-co-hexafluoropropylene (PVDF-HFP) shell[132]. **c** | Electrospun fibres containing bacterial microgels, made of multiple types of material[133]. Part **b** (top) adapted with permission from REF.[128], ACS. Part **c** adapted with permission from REF.[133], Wiley.

because the non-covalently stabilized fibres would dissolve in water prior to chemical crosslinking.

To prevent escape of the microorganisms from the fibres, core–shell fibres can be produced using coaxial nozzles. In these fibres, the core is formed by the microbial gel and the shell is made of a non-degradable polymeric matrix (FIG. 5b). Yeast cells (*Candida tropicalis*) can then be immobilized in core–shell fibres (10 µm diameter) consisting of a PVP core, prepared as an aqueous solution, and a polyvinylidenefluoride-co-hexafluoropropylene (PVDF-HFP) shell, prepared in an organic tetrahydrofuran/dimethylformamide (THF/DMF) solvent mixture[132]. PEG can be added as sacrificial material in the shell formulation to create pores (40 nm diameter) by dissolution and facilitate mass transport in and out of the core. Most yeast cells remain viable (95% viability for at least 17 days) and can grow and undergo budding, indicating that the PVP diffuses through the shell, thereby providing space for expansion of the yeast population.

Suspensions of bacterial microgels in a polymer solution can also be electrospun. The microgel provides a supportive environment for microbial survival and growth, and the surrounding matrix provides protection from the environment (FIG. 5c). For example, *E. coli* and *M. luteus* can be encapsulated in PVA, PVP, hydroxypropyl cellulose or gelatin microgels electrospun in DMF solutions of hydrophobic polymers (polyacrylonitrile, polystyrene, poly(methyl methacrylate) and poly(vinyl butyrate))[133]. Interestingly, the bacteria were able to survive short exposure to high temperatures (120–150 °C), which are required for the spray drying process to fabricate the microgel. The microgel protects the bacteria from the toxicity of DMF during electrospinning, and the resulting fibres contain viable bacteria within the microgels[133].

E. coli can also be immobilized inside or on the surface of electrospun fibres made of PEG–polylactide copolymers for drug delivery[134]. The bacteria, engineered to secrete enhanced green fluorescent protein as a model protein, can be either encapsulated within or immobilized onto the surface of the fibres, which results in viable bacteria, capable of growth and secretion of the protein. Extracts of the GRAS[34] microalga *Spirulina*, which produces several nutritive and therapeutic compounds, have also been electrospun in polyethylene oxide (PEO)[135], PCL[136] and silk fibroin[137] to fabricate tissue engineering scaffolds for stem cell, neuroregenerative and anti-thrombogenic applications, respectively. Although only lysed extracts of the microalga have been used in these applications, the electrospinning techniques could

also be employed for live cells. Indeed, electrospun living materials have so far been applied for bioremediation and catalysis, but the technology is sufficiently advanced to be applied for biomedical applications.

3D printing. 3D bioprinting of bacterial gels allows flexible fabrication of living devices with a wide range of geometrical design and structural and functional complexity (FIG. 6). 3D bioprinting requires suitable bioinks that allow mixing of the living component with the matrix precursor solution. In addition, the bioink should have sufficient viscosity to allow printing of defined structures and undergo rapid gelation after printing to retain the geometry of the printed thread[138]. Most importantly, the conditions of the bioprinting process need to ensure viability of the living component. A functional living ink (Flink) was developed by combining hyaluronic acid and κ-carrageenan as natural viscoelastic gel components with fumed silica as shear-thinning component[138]. A methacrylated version of hyaluronic acid allows chemical crosslinking of the bioink, stabilizing the printed structures[138]. Flink materials can be printed with high fidelity in various geometries, for example, for skin-graft applications, for which the printed Flinks contain viable bacterial cellulose-producing *Acetobacter xylinum* (FIG. 6a). Similarly, bioinks made of agarose solutions mixed with *B. subtilis* spores can be printed in the geometry of an artificial wound[24] (FIG. 6a). The living material perfectly seals the wound, and the bacteria release antimicrobial agents that kill the opportunistic pathogen *S. aureus*.

An acrylated derivative of Pluronic F-127 is also a popular bioink for the 3D printing of living materials[14,31]. This derivative shows thermo-responsive viscous gelation with a temperature increase from 4 °C to 37 °C, shear-thinning to aid extrusion and it can be chemically crosslinked to stabilize the printed structure. Using this material, stable, intricate and multifunctional 3D structures (FIG. 6b) can be printed with computational capabilities. For example, logic gate functions, such as NOT, AND, NAND and OR, can be implemented by printing fibres, from which chemical inputs (inducers, such as IPTG or rhamnose) diffuse into a living fibre containing bacteria that generate fluorescent proteins as an

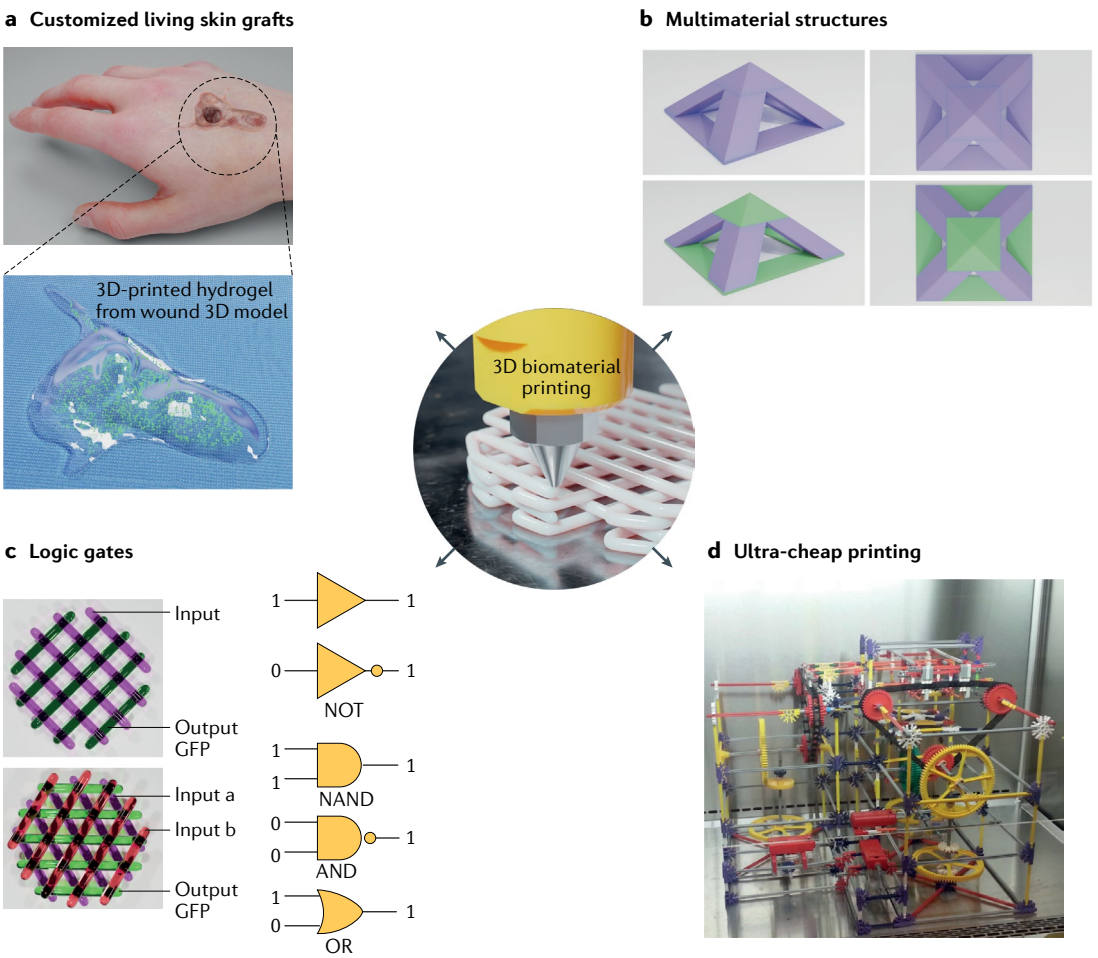

Fig. 6 | **3D printing of living materials. a** | 3D printing allows the fabrication of living materials in customized geometries, such as the shape of a skin wound[24]. **b** | Multimaterial structures can be 3D printed, enabling the incorporation of multiple bacterial strains[31]. **c** | Complex tasks, such as logic gate computations, can be programmed into living materials by 3D-printing multiple materials and strains[69]. **d** | The robustness of microorganisms allows the printing of living materials with cheap 3D printers, including home-made devices using K'nex[139,142,143]. GFP, green fluorescent protein. Parts **b** and **c** adapted with permission from REF.[14], Wiley. Part **d** reprinted with permission from TU Delft iGEM.

output based on genetically encoded logic functions[14] (FIG. 6c).

Alginate[139], nanocellulose and polyethylene glycol diacrylate (PEGDA)[140], as well as poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS)[141], have also been explored as bioinks. Using alginate as bioink, living materials containing microorganisms can be printed using simple and affordable (<500 USD per device) home-made printing devices[139,142,143] (FIG. 6d). Thus far, mainly additive manufacturing techniques have been applied for the 3D printing of living materials[6]. Therefore, current feature sizes remain large, in the range of 100 µm to several mm. Non-additive printing techniques could generate smaller structures; for example, multiphoton lithography can be applied to crosslink gelatin around bacteria, which results in the formation of micrometre-sized bacterial chambers. These chambers can be used to assess antibacterial responses in polymicrobial communities[144]. The range of bioink choices, bacterial functionalities, and dimensional and geometric features, as well as the affordability, make 3D printing an attractive technology for the design of living materials for biomedical applications.

The future of engineered living materials

Understanding microorganism–material interactions. The incorporation of microorganisms in materials[5] is usually characterized by microbial viability and functionality. In addition, material properties, such as elasticity, viscosity, porosity and degradation rates, are assessed for distinct applications and fabrication techniques. However, matrix–microorganism combinations are often selected by informed guesswork or trial and error. A better fundamental understanding of the interactions between microorganisms and their surrounding matrix would allow more insightful selection of both components.

Artificial biofilms have been designed as infection models to investigate microorganism–matrix interactions[145–148], mainly using agarose as a matrix. The polysaccharide agarose assembles into gels, which are stabilized by hydrogen bonds, similar to biofilm-produced matrices[145]. Using such artificial biofilms, variations in mechanical properties, such as stiffness, porosity, mass exchange rates and creep compliance, could be correlated with microorganism properties, such as growth rate, gene expression and antibiotic susceptibility[149]. For example, encapsulating *S. aureus* in agarose reveals that oxygen penetration, growth rate and antibiotic susceptibility are reduced if bacteria are located deep within the gel, further leading to an increase in the expression of a gene modulated by oxygen tension[149]. Therefore, microbial behaviour can be modulated by varying the mechanical properties of the matrix.

In living materials, a range of matrix components are used with different possibilities for crosslinking through covalent, van der Waals or hydrophobic interactions or chelation[5]. Gels made of triblock copolymers (for example, Pluronic F-127) can be applied to investigate microbial morphologies, for example, of yeast, by optical and scanning electron microscopy[150]. The bacterial hydrogels, which are formed through non-covalent interactions, can be 3D printed and then covalently crosslinked for stabilization. Yeast colonies encapsulated in these hydrogels are bigger in the periphery of the material than in the centre, most likely owing to the greater availability of oxygen and nutrients in these regions[151]. Interestingly, colony morphology appears different in hydrogels made of triblock copolymers with an ABA structure (Pluronic F-127-based) than in polymers with a BAB structure (poly(isopropyl glycidyl ether-*stat*-ethyl glycidyl ether)-*block*-poly(ethylene oxide)-*block*-poly(isopropyl glycidyl ether-*stat*-ethyl glycidyl ether) dimethacrylate (PGE-DMA)), where A are hydrophilic blocks and B are hydrophobic blocks[150]. Yeast colonies in the ABA hydrogels are predominantly spherical (with diameters of 90–250 µm at 72 h), whereas those in BAB gels also show elliptical or spindle-shaped morphologies. Although the mechanism underlying the formation of different morphologies remains elusive, differences in the micellar structure of the polymers within the gels may play a role. Interestingly, encapsulation results in smaller cell sizes compared with the sizes of cells grown in culture[150]. Furthermore, cells are smaller in gels with higher storage modulus. In addition, cell colonies in the ABA hydrogels are covered by a thin organic layer (100–160 nm thickness), which is not present in the BAB hydrogels[150], which may also be related to differences in micellar identities between the two types of polymer, possibly resulting in different types of interaction with the cells prior to covalent crosslinking[150]. To gain better insight into the specific interactions between the living and material components and to enable well-informed design strategies for specific applications, more systematic investigations will be required.

Biocompatibility and immunogenicity testing. Composite materials that contain viable microorganisms and polymeric matrices pose a challenge in terms of biocompatibility. Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy[152]. In this Review, we refer to the ability of the entire living material, including the synthetic material and bacteria, to exert a specific role without causing adverse effects to the patient[153].

The study of biocompatibility is well established for biomaterials, with a number of protocols defined in the literature (for example, ISO 10993 (REF.[154]) for haemocompatible materials and other medical devices). For example, to assess biocompatibility in vivo, the foreign body reaction[155] needs to be assessed. For living materials, the biodistribution of bacteria in the host needs to be carefully investigated, and biocompatibility strongly depends on the species, the specific strains, the site of implantation and the desired role of the living material[26]. The immunogenicity of several major species and genera of bacteria has already been described[156–158]. Several lactic-acid-producing bacteria have GRAS status, which is an FDA classification applied to food additives that are considered safe for human consumption, including

Lactococcus, *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc* and *Bifidobacterium*[159]. *Lactococcus* has also been used as a vaccine delivery vehicle[160,161], owing to its low immunogenicity and its ability to produce and display heterologous proteins. For example, *L. lactis* expressing recombinant antigens can be delivered as mucosal immunogenic live vaccine[162]. *E. coli* strains can be classified according to their pathogenicity in different 'pathotypes'[163], that is, strains that cause a common disease and share a common set of virulence factors. For example, some pathogenic strains cause enteric or diarrhoeal infections, sepsis and urinary tract infections, whereas other strains (K12 (REF.[164]), B, C, W (REF.[165]) and Nissle[26]) are known laboratory workhorses with very limited or no pathogenicity and a Class I biosafety level[163].

Biosafety concerns. Engineered living materials contain viable and, in some cases, genetically engineered cells. Therefore, it is important to acknowledge and prevent the environmental risk associated with their interaction and release into the environment or in the body. Dense matrices can be used to prevent proliferation and leakage of cells from biomaterials, and genetic kill switches can be implemented to prevent cell viability outside a controlled environment. Microevolution is also a concern for microorganisms embedded in biomaterials. Prokaryotes can undergo rapid genetic and phenotypic changes as they multiply from a single cell, which can give rise to a variety of clones with more virulent traits. Microevolution takes place in response to local environmental changes[166] through point mutations, genetic rearrangements, conjugation, transduction and transformation. Transposon mobility and horizontal gene transfer can then lead to the evolution of new species[167], which may affect applications of living biomaterials.

Synthetic biology strategies are emerging to prevent or decelerate the evolutionary failure of genetic circuits[168–170], which could also be implemented in living materials. In particular, the possibility of gene transfer could be reduced by engineering genetic circuits within the chromosome or within complementary plasmid–bacteria systems that are encoded with plasmid-retention pressure conditions[171]. This can be achieved by engineering auxotrophy or other genetic defects and by supplementing a missing gene or essential metabolite. For example, a *L. lactis* strain lacking thymidylate synthase is thymine-dependent, which makes it unviable outside a controlled environment, in which thymine is supplied[172]. In such strains, a plasmid containing thymidylate synthase would be naturally retained when thymine is not available from external sources. One minor drawback to this strategy is that the modified bacteria need access to this metabolite in the environment. Auxotrophic systems can be synthetically designed; for example, *E. coli* can be genetically engineered to be dependent on synthetic amino acids to remain viable[173], by converting the TAG stop codon into a codon for a synthetic amino acid through an orthogonal translation auxotrophy.

Active containment strategies can be achieved by controlling the expression or inhibition of a lethal function through sensory systems that recognize physical or chemical signals in the local environment[174]. For example, the expression of a nuclease in *Serratia marcescens* can be induced by arabinose[175]. The nuclease degrades the bacterial genome and, thus, inactivates the bacteria. Alternatively, safeguard systems have been engineered, namely, the Deadman and Passcode kill switches. The Deadman kill switch uses a genetic toggle switch; here, viability of the bacteria depends on the presence of anhydrotetracycline (ATc), a TetR inhibitor. In the absence of ATc, toxin genes are derepressed, which causes death of the microorganism. The Passcode kill switch is based on gene activation of a toxin, which degrades proteins essential for the viability of the cell. This switch is controlled by three separate inputs and requires the presence of two inputs and the absence of one to repress toxin production. Otherwise, the toxin will be produced to kill the bacteria. Combining both switches provides a secure containment strategy, because cell viability requires the presence of two molecules; the lack of one or both molecules triggers the suicide switch. To avoid any possible impact of mutations on these systems, multiple copies of the switch can be used to provide fail-safe design[176].

These synthetic biology strategies can address safety concerns of living therapeutics, for which bacteria are freely administered to the body. Alternatively, safety issues can be addressed in living materials through material design aspects. For example, cells can be encapsulated in a way that prevents their proliferation and dispersal into the host organism for in vivo applications. Physical containment can be achieved by using highly crosslinked gels with a pore size smaller than the diameter of the bacteria, by surface modification and/or covalent binding of the bacteria to the scaffold[172,177]. In addition to physical containment, a matrix can be applied that slows down bacterial leakage through adhesive interactions[102]. Degradable drug-release matrices are also being explored for the design of living materials. For example, the material could be designed to release antimicrobials that kill bacteria as it degrades, thereby establishing a defined lifetime for the device. Analogously, matrices could be designed to release antimicrobials in case of material breakage, to prevent the escape of bacteria into the environment.

The use of living microorganisms as therapeutics has mainly been limited to probiotics[178] thus far, for various applications, from acute gastroenteritis to intestinal neoplasia. Probiotics are usually marketed as dietary supplements and, thus, commercialization requirements are less stringent than for pharmaceutical or biological products. Probiotics have been approved for the treatment of diarrhoea[179,180], chronic pouchitis, a complication of inflammatory bowel disease[181,182], and non-intestinal diseases, such as atopic eczema[183,184], as well as psychiatric disorders, such as depression[185].

Outlook

Engineered living materials have the potential to revolutionize the field of dynamic biomedical materials. This nascent field has already shown promising results for wound healing, drug delivery and stem cell engineering. Various fabrication approaches have been applied to

engineer living materials, including 3D printing, microfluidics, electrospinning and microencapsulation, to encapsulate genetically engineered microorganisms that can sense and respond to changes in the environment, such as pH, temperature and biochemical composition. The response of living materials (for example, release of drugs or proteins) can be designed to be triggered by light or small molecules in culture media. However, challenges remain to develop living materials that can 'feel' their environment and respond to it. For example, living materials could be designed for stem cell engineering. Bacteria-laden hydrogels could respond to changing oxygen, tension or chemical gradients by secreting growth factors and cytokines that fulfil the metabolic demand of stem cells. Such living materials could be applied to maintain a stem-cell-like niche or engineered to respond to an insult (that is, self-healing materials) to provide different biochemical environments in response to the needs of the cell population.

Engineered living materials have mainly been developed for in vitro applications thus far, with a few exceptions of materials applied for wound healing. We envisage that living materials will also be part of advanced in vitro models, in which materials need to have dynamic properties to recapitulate the extracellular matrix of tissues, for example, to control cell differentiation upon regenerative demand. More importantly, living materials may be used for therapeutic applications in the future; for example, living materials could be engineered as drug-eluting devices that can detect glucose levels and respond with insulin secretion, providing a permanent therapeutic solution to diabetes. However, clinical translation of living materials requires regulatory approval, which is challenging, owing to the presence of microorganisms. In particular, potential release of bacteria needs to be prevented to avoid systemic distribution of microorganisms. Moreover, living materials need to be tolerated by the immune system; that is, the microorganisms need to be shielded from the immune system. Finally, microorganisms may also be engineered to contain an externally controllable switch-off function to ensure biosafety.

To advance the field of engineered living materials, several key areas need to be addressed. Microorganisms need to be genetically engineered to perform more complex functions (synthetic biology); synthetic materials need to be optimized that can encapsulate microorganisms and provide an adequate environment for the microorganisms to perform their function, including adequate oxygen permeability, porosity and stability; and manufacturing technologies need to be developed that allow the combination of microorganisms with synthetic materials. We envisage that additive manufacturing technologies and bioink development will be particularly relevant for the progress of engineered living materials.

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