

Review

Biological Engineered Living Materials – growing functional materials with genetically-programmable properties

Charlie Gilbert, and Tom Ellis

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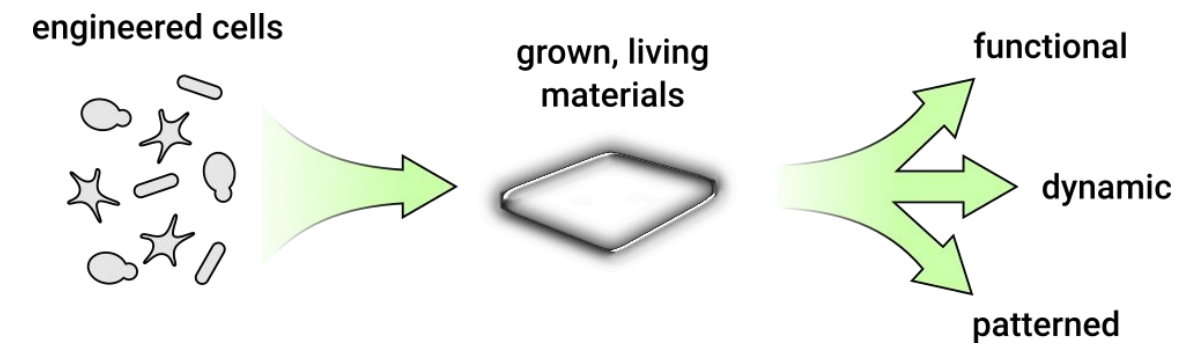
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Biological Engineered Living Materials – growing functional materials with genetically-programmable properties

Charlie Gilbert^{1,2} and Tom Ellis^{1,2}

- 1. Centre for Synthetic Biology, Imperial College London, London SW7 2AZ, UK
- 2. Department of Bioengineering, Imperial College London, London SW7 2AZ, UK

Correspondence to: t.ellis@imperial.ac.uk



Natural biological materials exhibit remarkable properties: self-assembly from simple raw materials, precise control of morphology, diverse physical and chemical properties, self-repair and the ability to sense-and-respond to environmental stimuli. Despite having found numerous uses in human industry and society, the utility of natural biological materials is limited. But, could it be possible to genetically program microbes to create entirely new and useful biological materials? At the intersection between microbiology, material science and synthetic biology, the emerging field of biological Engineered Living Materials (ELMs) aims to answer this question. Here we review recent efforts to program cells to produce living materials with novel functional properties, focussing on microbial systems that can be engineered to grow materials and on new genetic circuits for pattern formation that could be used to produce the more complex systems of the future.

Many biological materials are familiar to us from the natural world – materials like wood, shell or cotton. From the standpoint of materials science, these materials exhibit some impressive traits. Firstly, starting from single cells, organisms can proliferate and direct the conversion and accumulation of simple raw materials to form large, complex materials. Secondly, following pre-programmed genetic rules, the cells that orchestrate the synthesis of these materials exert an incredible degree of control over the morphology of the structures they form over multiple length scales. Thirdly, over the course of evolution, natural biological materials have acquired a staggering range of properties – from electrical conductivity to strong underwater adhesion to thermoplasticity. Fourthly, natural biological materials are not inert, instead, the cells that produce these materials and remain associated with them are able to sense changes in their environment and respond appropriately to modify the material properties in some way. Finally, biological material assembly can occur under mild conditions, driven by sustainable energy sources and raw materials, and resulting in the production of biodegradable products.

While materials like wood, cotton, leather and silk have found many uses in human industry and society, the properties of natural biological materials are constrained to those selected for by evolution. The emerging field of engineered living materials (ELMs) aims to meet this limitation by recreating and engineering the natural processes of biological material assembly within designed materials. Broadly, ELMs are defined as materials in which living cells form an integral part^{1–3}. While current definitions of ELMs encompass 3D-printed and biohybrid materials, consisting of both biological and synthetic components³, a long-standing aim is to create ELMs in which living cells are engineered to autonomously self-assemble entire materials. We refer to this subset as 'biological ELMs', where the material assembly process occurs through biological self-assembly. Typically, these materials are made from biological macromolecules produced by cells, but these materials may also contain inorganic components if accumulated or deposited biologically; for example, by biomineralization, as seen naturally in bone and shell. While the broader field of ELMs has been excellently reviewed elsewhere³, in this review, we focus specifically on biological ELMs. The goal of the field of biological ELMs is to genetically engineer microbes and other biological systems to produce novel, useful materials with programmable properties. Achieving this major challenge could lead to a new paradigm for material production, taking

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advantage of the remarkable properties of living biology. However, at the same time, developing new and useful biological ELMs presents a number of complex challenges. The genetic rules governing the functional properties and morphogenesis of biological materials are still being uncovered. And rationally-engineering these processes requires the use of well-characterised genetic parts and circuits from the field of synthetic biology. Indeed, efforts to develop biological ELMs from microbes using synthetic biology approaches have only recently begun in earnest. Here we review the emerging topic of biological ELMs produced by microbes, and discuss the approaches and systems being used to engineer this new class of materials. We first highlight two model biological ELM systems – bacterial biofilm amyloids and bacterial cellulose – and examine how they have been engineered to create novel, functional materials. We then discuss various approaches to genetically-program pattern formation to control material morphology in microbes, highlighting important related work illustrated in mammalian cells. Finally, we review more complex biological systems which may emerge as the biological ELMs of the future.

Bacterial biofilm amyloids and curli fibres

Many pioneering efforts to develop biological ELMs have focussed on engineering *E. coli* biofilms. In this section we discuss some of the natural properties of these simple, genetically-tractable model systems and review recent efforts to genetically-program novel, useful material properties in this emerging class of biological ELMs.

Structure and properties of bacterial biofilm amyloids

Amyloids are fibres formed by a broad group of proteins and are defined by a common β -sheet rich protein structure, the cross- β strand^{4,5}. Although originally isolated because of their connection to various human disease states, amyloids are found in diverse organisms, from bacteria to humans⁶, and perform numerous functional roles as toxins, adhesins, surface property modifiers and more⁷. The cross- β strand amyloid fibre structure consists of a flattened coil of β -strands that form a continuous hydrogen-bonding network running the

length of the fibre. This extended hydrogen-bonding network results in the remarkable material properties of amyloid fibres – such as high tensile strength and Young's modulus⁸ – as well as their exceptional resistance to degradation by proteases and denaturation⁹. Many bacteria take advantage of the physical properties of amyloids to build biofilms. Bacterial biofilms are essentially composite materials, consisting of living cells that produce and become embedded within an extracellular matrix. The extracellular matrix itself is composed, chiefly, of polysaccharides, nucleic acids and amyloid proteins. In the natural environment, bacteria produce biofilms to enable attachment to surfaces and long-term persistence¹⁰.

The best studied of the bacterial biofilm amyloids are curli fibres (Figure 1a). Curli fibres, also known as curli pili, are produced by a number of species of the family *Enterobacteriaceae*, most notably by *E. coli*. Because of their known roles in bacterial pathogenesis, the biogenesis of curli fibres has been extensively studied (Figure 1b). Briefly, a number of different proteins are expressed from the curli (*csg*) operon and secreted into the *E. coli* periplasm. Here they form a pore in the outer cell membrane through which the major curli subunit CsgA, a 13 kDa protein, is secreted¹¹. Once outside the cell, the cell-surface CsgB protein nucleates the conversion of soluble CsgA to the amyloid form and polymerisation proceeds to create curli fibres¹² (Figure 1c).

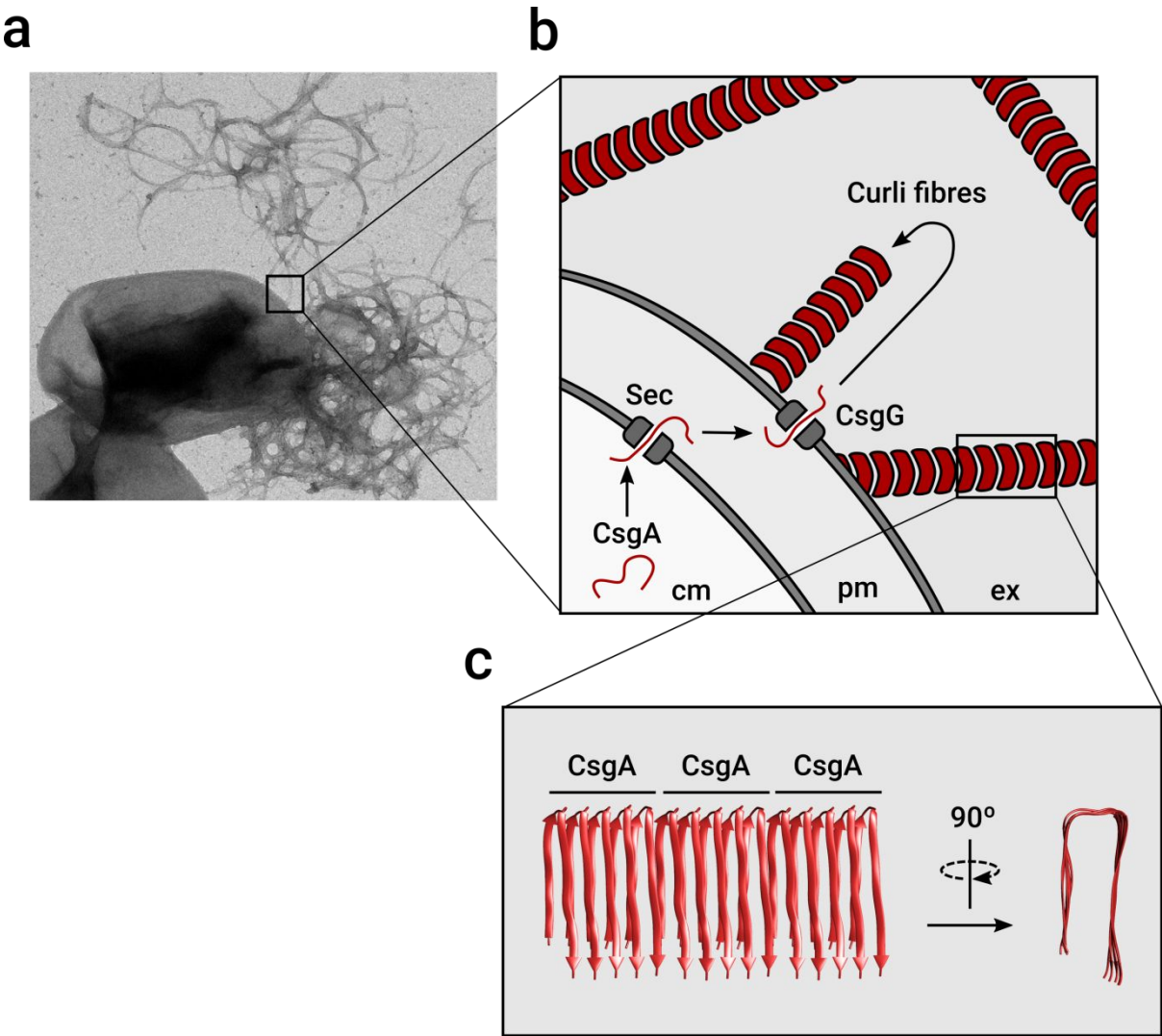


Figure 1 *E. coli* curli fibre structure and biogenesis. (a) TEM image of a curli-producing *E. coli* bacterium. Curli fibres are visible as hair like aggregated structures in the extracellular environment. Image reproduced from Van Gerwen et al.¹⁴⁰. (b) Schematic showing curli fibre biogenesis. CsgA monomers are secreted from the cytoplasm (cm) to the periplasm (pm) via the Sec translocon and then from the periplasm to the extracellular environment (ex) via an outer membrane pore formed by the CsgG protein. Once outside the cell, CsgA monomers polymerise to form curli fibres. (c) Curli fibre structure. Curli fibres consist of stacked CsgA monomers. Individual monomers adopt the cross β -strand structure that is characteristic of amyloids. Each monomer consists of five turns of the cross β -strand structure. Individual monomers stack end-to-end, resulting in a continuous network of hydrogen-bonds along the fibre axis.

***E. coli* curli biofilms as biological ELMs**

The combination of their polymeric nature, impressive mechanical properties and extracellular production from a genetically-tractable host make *E. coli* curli fibres ideal targets for biological ELM development. In 2014, two landmark studies took advantage of the favourable properties of *E. coli* bacterial biofilms to create genetically-programmable functional materials. Both studies relied on the fact that short polypeptide sequences could be fused to the C-terminus of the CsgA monomer without impacting curli fibre formation (Figure 2a). This ability to incorporate heterologous peptides into curli fibres offered a simple handle to load functional modules onto the curli fibres (Figure 2b-d).

In the first of these studies, *E. coli* were engineered to allow inducible expression of the curli fibres¹³. To enable on-demand production of curli fibres, an *E. coli* strain was engineered to constitutively express the genomic *csg* operon required for curli fibre biogenesis but lack the native *csgA* gene. Fibre formation could then be initiated by inducing expression of plasmid-borne *csgA*. To modify the functional properties of curli fibres, short peptide tags were fused to the C-terminus of the CsgA monomers, including: the hexa-histidine tag (CsgA_{His}), the SpyTag (CsgA_{SpyTag}) and the FLAG tag (CsgA_{FLAG}). Leveraging these functional modules, chemical species were added that bound the peptides tags, creating biofilms functionalised with gold nanoparticles and quantum dots. In addition, by co-culturing *E. coli* strains producing different CsgA monomers, two-component curli fibres were generated consisting of alternating stretches of each monomer. By applying gradients of chemical inducers controlling the expression of each protein, the patterning of different CsgA monomers within curli fibres could be controlled over multiple length scales. Finally, this system was used to demonstrate that living cells can be engineered to autonomously pattern a biological material. To achieve this, a CsgA-secreting strain was engineered to constitutively produce a quorum-sensing molecule, acyl homoserine lactone (AHL), which could activate the expression of CsgA_{His} from a second strain. When co-cultured, these strains initially produced curli fibres consisting predominantly of CsgA monomers, but over time, as AHL accumulated in the culture medium, an increasing proportion of CsgA_{His} monomers were incorporated. This engineered cell-cell communication system therefore enabled genetically programmed, autonomous control of the patterning of curli fibres over time. The authors illustrated the

utility of their system by producing functional composite materials, such as gold nanowires, nanorods and conductive biofilms.

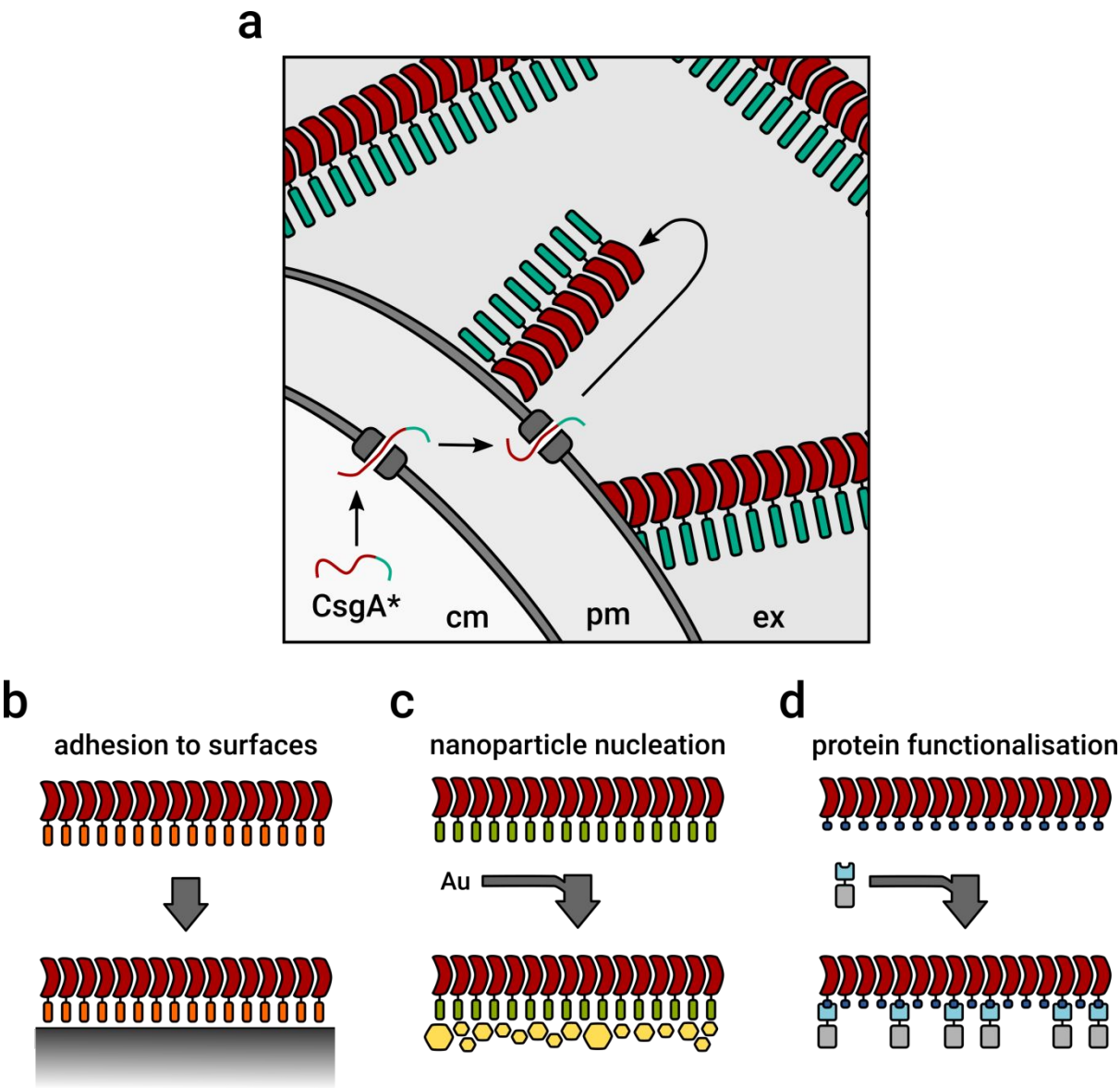


Figure 2 Engineering curli fibres to create functional biological ELMs. (a) Modifying the CsgA sequence enables curli fibre functionalisation. CsgA monomers to which peptide sequences have been fused (CsgA*) can be heterologously expressed. Depending on the sequence of the additional peptide sequence, CsgA* monomers can be correctly secreted and assembled to form extracellular curli fibres displaying user-defined functional modules. A number of heterologous peptides can be fused to CsgA monomers to create functionalised curli materials, including (b) peptides mediating adhesion to surfaces, such as steel, (c) peptides that can direct and modify the mineralisation of gold nanoparticles and (d) peptides enabling downstream conjugation of functional protein modules, such as industrial enzymes.

Following this, a second study described how a variety of additional novel functional properties can be genetically programmed into *E. coli* biofilms by fusing an expanded set of peptide domains to the CsgA monomer¹⁴. The system, which the authors call biofilm-integrated nanofiber display (BIND), again relies on the fact that peptide domains can be fused to the CsgA monomer without abolishing curli fibre formation. After screening for the optimal fusion site on the CsgA monomer, the authors showed that a panel of peptides, able to bind a variety of species – including ice crystals, carbon nanotubes and magnetite – could be fused to the CsgA monomer without disrupting curli fibre formation. Several candidates were then selected and shown to confer new functional properties to the engineered biofilms: the A3 peptide enabled silver nanoparticle capture, a metal-binding domain allowed stable adhesion to stainless steel and the SpyTag peptide was used to covalently capture tagged fluorescent proteins. Of particular interest, decoration of CsgA with the short SpyTag peptide enables covalent attachment of its binding partner, the SpyCatcher protein. Together the SpyTag-SpyCatcher pair autocatalyse the formation of an intermolecular covalent bond between two amino acid side chains¹⁵. The authors showed that curli fibres could, therefore, be functionalised in a modular manner, simply by externally adding a protein of interest fused to the SpyCatcher domain.

This SpyTag-SpyCatcher approach has since be extended to create catalytic biofilms. Biofilms were functionalised by expressing SpyTag-displaying curli fibres and incubating with a pre-purified recombinant α -amylase-SpyCatcher fusion protein¹⁶. In addition, taking advantage of the natural resilience of biofilms to environmental challenges, the authors showed that catalytic biofilms remained active following exposure to a range of pH and organic solvent conditions. A subsequent study built on this approach further by generating a panel of orthogonal protein-protein conjugation domains, as alternatives to the SpyTag-SpyCatcher¹⁷. Using this expanded set of tools, the authors showed that curli fibres could be conjugated to multiple tagged recombinant proteins obtained directly from crude *E. coli* cell lysates. This system was leveraged to create two-enzyme biocatalytic biofilm materials, able to perform a stereoselective ketone reduction transformation, an industrially-relevant reaction. Importantly, to take these bacterial biofilm materials closer to industrial applications, a simple, versatile method for scalable production and purification of functionalised curli fibres was recently described¹⁸. This approach enables isolation of tens of milligrams of curli fibre

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3 material per litre of bacterial culture and simple production of gels and films. However, one
4 conceivable limitation of these approaches is the need for prior production of the
5 functionalising module – the SpyCatcher-enzyme fusion protein. A system directing
6 simultaneous, *in vivo* production of curli fibres and functionalising modules would enable the
7 useful traits of living materials to be leveraged within these systems. For instance, the
8 patterning and relative abundance of different enzymes conjugated to curli fibres could be
9 controlled using genetic circuits. In the future, *in vivo* production of functionalised curli fibres
10 might be achieved, for example, by engineering co-secretion of SpyCatcher fusion proteins.

11
12 One area that has received particular focus is the creation of electrically-conductive curli
13 fibres. Expanding on previous work, a panel of inorganic-material-nucleating peptides were
14 fused to CsgA and shown to seed the nucleation and formation of gold nanoparticles and,
15 therefore, to create electrically-conductive biofilms¹⁹. Interestingly, particular peptides
16 directed the mineralisation of gold nanoparticles with different size distributions. Exploiting
17 this phenomenon, the choice of nucleating peptide was shown to enable tuning of the
18 electrical conductivity of the engineered biofilms. An alternative approach was inspired by
19 the naturally-occurring conductive protein nanowires produced by the bacterium *Geobacter*
20 *sulfurreducens*²⁰. *G. sulfurreducens* produces extracellular protein filaments, in which
21 continuous stretches of aromatic amino acid side chains conduct electrical charge along the
22 length of the filaments^{21,22}. Inspired by this natural system, aromatic amino acids were fused
23 to the C-termini of CsgA monomers, to create stripes of aromatic side chains running along
24 the length of the resultant curli fibres. The authors found that the resulting curli fibres and
25 bacterial biofilms showed a mild, but appreciable increase in electrical conductivity²³. In an
26 extension of this approach, a recent report installed aromatic amino acids directly into the
27 CsgA monomer sequence to create conductive curli fibres, removing the need for fusion of
28 additional amino acids²⁴. Further, the authors utilised the previously-described scalable
29 purification process¹⁸ to generate macroscopic, conductive curli gels and films. While the
30 conductivity of these modified curli fibres remains lower than that of naturally-occurring
31 *Geobacter* nanowires, the ease of production and relatively large yields obtained could
32 enable applications in bioelectronic systems such as microbial fuel cells, biosensors and
33 electrogenetic systems.

Another study fused mussel foot proteins (Mfp) to CsgA to create a strong, underwater adhesive material²⁵. Mfps are a family of proteins secreted by mussels to form the holdfast, or byssus, with which they adhere to solid surfaces in marine environments^{26–28}. Two Mfp proteins, Mfp3 and Mfp5, were genetically fused to CsgA and expressed intracellularly in *E. coli*. The two fusion proteins, CsgA-Mfp3 and CsgA-Mfp5, were then purified and enzymatically-modified, to recreate cross-linkages formed in the natural mussel byssus. Remarkably, the resultant fibres exhibited underwater adhesion energy – a measure of the energy required to separate two adhered materials – greater than all previous bio-derived and bio-inspired protein-based underwater adhesives. Importantly, this example does not strictly meet the definition of biological ELMs since its production requires extensive external intervention. However, it clearly illustrates how modular, functional protein domains can be fused to curli fibres to create engineered biological materials with novel properties, an approach that could be utilised in true biological ELM systems.

Many of the studies described here use a modular approach to programmably functionalise curli fibres, in which users can pick and choose protein modules with which to decorate curli fibres. A potential limitation of this approach is the requirement for fused protein sequences to retain their function without interfering with the CsgA protein secretion and amyloid assembly. However, it has been shown that protein sequences of up to at least 260 amino acids can be fused to CsgA without abrogating curli fibre assembly²⁹. In situations where functional protein modules cannot be fused directly to the CsgA monomer, coproduction of SpyTag-displaying CsgA monomers and SpyCatcher-displaying functional protein modules offers an alternative solution.

In a remarkable study leveraging another of the advantages of biological ELMs, *E. coli* biofilms were engineered to act as environmentally-responsive bioremediatory materials³⁰. Inspired by a previous report suggesting curli fibres play a protective role in natural biofilms by absorbing the heavy metal pollutant mercury³¹, the authors engineered an *E. coli* strain to produce curli fibres in response to environmental mercury. The CsgA monomer was expressed from a mercury-inducible promoter which becomes active in response to environmentally-relevant concentrations of mercury. The resultant curli fibres were able to efficiently bind and sequester mercury, acting as a sponge to mop up the heavy metal. Notably, this system takes advantage of the ability of living cells to sense and respond to

their environment; the bioremediatory biofilm material is only produced in response to the detection of the heavy metal pollutant. As the authors note, in the future, *E. coli* strains engineered to produce curli fibres in response to a variety of pollutants could be deployed into the environment to sequester multiple pollutants or toxins.

The future prospects of biofilm biological ELMs

While impressive progress has been made to create *E. coli* biofilm ELMs, there is great scope to expand these approaches by engineering alternative bacterial biofilms. For instance, the Gram-positive bacterium *Bacillus subtilis*, a standard synthetic biology host organism, produces biofilms containing fibres formed from the TasA protein³². Although initially thought to adopt the characteristic cross- β strand amyloid structure, recent evidence suggests that TasA fibres are in fact composed of a distinct, non-amyloid structure³³. It has already been shown that antigenic peptides and a full-length fluorescent protein could be fused to TasA to functionalise biofilms³⁴. Notably, *B. subtilis* is one of the preferred host organisms for secretion of recombinant proteins³⁵. *B. subtilis* may therefore represent an ideal future candidate for simultaneous secretion of protein fibres and functional proteins, enabling *in vivo* production of functionalised biofilms. Interestingly, *B. subtilis* biofilms exhibit some remarkable natural properties. Firstly, they are highly hydrophobic – surpassing even the water repellence of Teflon³⁶ – a property conferred by another secreted protein, BslA^{37,38}. Secondly, it has recently been shown that the growth of *B. subtilis* biofilms oscillates in highly-ordered manner³⁹. These oscillations were shown to be orchestrated by an unprecedented electrical cell-cell communication system based on K⁺ ion flux across the *B. subtilis* cell membrane⁴⁰. In the lab, this electrical signalling system has been shown to enable recruitment of motile cells to biofilm communities⁴¹ and to allow long-range communication between two distinct biofilms⁴². This new paradigm for bacterial cell-cell communication may therefore represent a useful target for future engineering of *B. subtilis* ELMs. From the single example of *B. subtilis*, it is clear that alternative bacterial biofilm systems have great potential to expand the scope of biological ELMs. Since homologues of the curli operon have been found in numerous species of bacteria, spanning at least four distinct phyla⁴³, there may be much more to learn.

Bacterial cellulose

Another exciting biological ELM is bacterial cellulose (BC). Due in part to the impressive natural properties of BC, there have been growing efforts to genetically-engineer BC-producing bacteria to produce novel, useful biological materials. In this section, we describe the important properties of BC and BC-producing bacteria and outline various efforts to genetically-engineer BC-bacteria and other microbes to create a new class of biological ELMs.

The structure and properties of bacterial cellulose

Cellulose, a polysaccharide composed of β -(1 $\rightarrow$ 4)-linked glucose units, is the most abundant biopolymer on the planet⁴⁴. While best known as the major structural component of many plant tissues, several species of bacteria are also capable of producing cellulose⁴⁵. Plant-derived cellulose has been used by humans for centuries as a sustainable source of clothing, construction materials, paper and so on. But BC exhibits some additional, uniquely advantageous physicochemical properties, such as high crystallinity, high tensile strength, high purity, ultrafine network architecture and biocompatibility⁴⁶. As a result, BC has garnered much interest as a feedstock material for industrial applications.

The most prodigious producers of BC are Gram-negative acetic-acid bacteria (AAB), in particular the genera *Gluconacetobacter* or *Komagataeibacter*⁴⁷, hereafter referred to generally as BC-producing bacteria. Thanks to interest in BC, there has been an increasing body of research into the basic cellular physiology of BC-producing bacteria, focussing mainly on *Gluconacetobacter xylinus*. The predominant native ecological niche of *Komagataeibacter* and *Gluconacetobacter* species is as colonisers of fruit. Here, the production of BC serves to form a biofilm that protects cells from desiccation and UV damage⁴⁸. By contrast, when grown in non-native conditions in the lab, BC-producing bacteria are able to produce macroscopic floating mats of BC. Under static growth in liquid culture, BC-producing bacteria grow predominantly at the air-water interface, where aerobic growth is best supported. As they grow, BC-producing bacteria synthesise and secrete chains of cellulose, which remain attached to the cell surface. The result, referred to as a pellicle, is a floating mat of intertwined cellulose fibrils, within which individual cells are embedded

(Figure 3a, 3b and 3c). Remarkably, BC-producing bacteria are able to synthesise extracellular cellulose reaching yields in excess of 10 grams per litre⁴⁹.

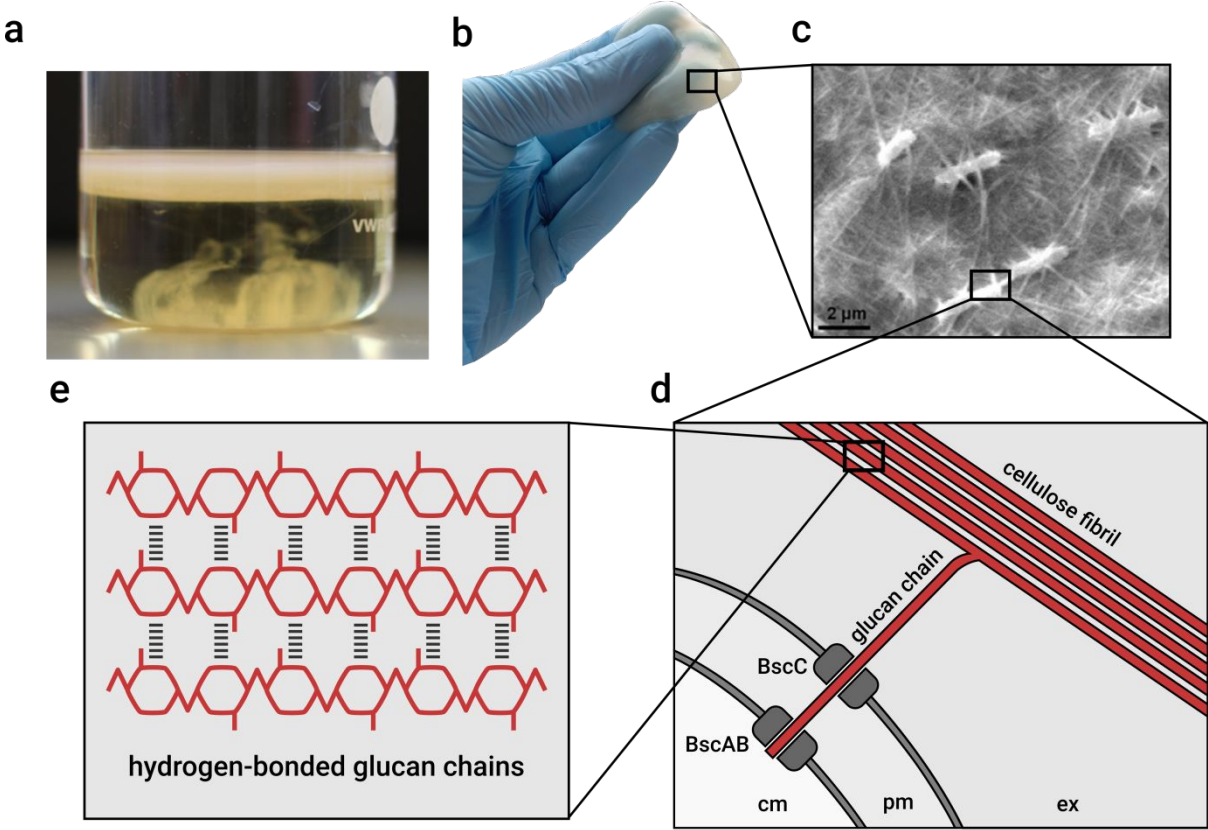


Figure 3 Bacterial cellulose structure and biogenesis. (a) When grown under static conditions in liquid culture, BC-producing bacteria form a floating layer of cellulose (white), known as a pellicle. (b) An isolated BC pellicle. Pellicles in their native state are rubbery and tough. (c) SEM image of the surface of a pellicle, reproduced from Florea et al.⁸³. Bacterial cellulose fibrils form an interwoven network into which individual bacteria become embedded. Fibres in native pellicles are not as tightly compacted as seen here under vacuum. (d) Monomers of glucose are simultaneously polymerised and secreted by the macromolecular cellulose synthase complex. Outside the cell, individual glucan chains are bundled together to form ribbon-like BC fibrils. Cytoplasm (cm), periplasm (pm), extracellular environment (ex). (e) Glucan chains are bonded to one another through networks of hydrogen-bonds.

Although still not fully understood, a great deal of research has focused on the molecular details of BC biosynthesis. Cellulose biosynthesis is fed by intracellular UDP-glucose monomers. Cellulose synthesis takes place within membrane-spanning, multi-subunit cellulose synthase complexes. Here, UDP-glucose monomers are added to the growing cellulose chain, while the entire chain is simultaneously translocated across the inner and outer cell membranes (Figure 3d)⁵¹. Although numerous proteins play a role in orchestrating BC synthesis and assembly, the *bcsABCD* operon encodes four essential proteins that make up most of the cellulose synthase complex⁴⁹. BcsA plays a number of roles: it forms part of the pore across the inner membrane, catalyses the polymerisation of UDP-glucose units and enables allosteric regulation of cellulose synthesis by cyclic-di-GMP. BcsB also contributes to the formation of the inner membrane pore. BcsC, a periplasmic protein, is believed to form β -barrel pore in the outer membrane, through which the glucan chain is threaded. Lastly, a second periplasmic protein, BcsD, is believed to be responsible for the correct orientation of cellulose synthase complexes relative to one another.

These remarkable molecular machines are able to polymerise up to 200,000 molecules of glucose per second⁵². Electron microscopy examination of BC-producing cells revealed the presence of ~50 pore-like structures arranged in-line with growing BC fibrils^{53,54}. It is believed that these pore-like structures are themselves composed of multiple cellulose synthase complexes, each secreting a single glucan chain. In a process that is yet to be fully elucidated, a single cell is able to simultaneously polymerise, secrete and bundle together around 1000 individual glucan chains into a hierarchy of intermediate structures, finally producing ribbonlike cellulose fibrils, 50-80 nm in width and 1-9 μ m in length^{50,55}. As with other forms of cellulose, individual glucan chains are strongly held together by a combination of van der Waals' forces and hydrogen-bonds between glucose hydroxyl groups⁵⁶ (Figure 3e). The resulting BC material is comparatively pure, lacking substances typically associated with plant cellulose such as hemicellulose, pectin and lignin. Owing to its high degree of crystallinity – that is, the regular arrangement of glucan chains – BC exhibits excellent mechanical properties: single BC nanofibers have tensile strength estimated to be ~1500 MPa⁵⁷ and Young's modulus of ~114 GPa⁵⁸. Further, BC is biocompatible – meaning it is not toxic to human tissue – and biodegradable. Given its desirable material properties, BC has been developed for a variety of potential commercial applications: as stabilisers for

foams and emulsions, as scaffolds for tissue engineering, as separators for batteries, and as new fibres for textiles. In fact, there are several commercially-available bacterial-cellulose based products, such as BioProcess®, marketed by Fibrocel Brazil, which is a wound-dressing used in the treatment of burns and ulcers⁵⁹ and bacterial cellulose-derived acoustic diaphragms for use in headphones from Sony Corporation⁶⁰.

Bacterial cellulose as a biological ELM

There have been numerous efforts to develop novel BC-based materials with improved material properties. One common approach to develop new BC-based materials is to blend BC with another biological macromolecule to create a composite. In fact, many biological species have been used to modify BC material properties, including spider silk⁶¹, gelatin⁶², zein⁶³, collagen⁶⁴, hyaluronan⁶⁵, alginate⁶⁶, heparin^{67,68}, antimicrobial peptides⁶⁹ and growth factors^{70,71}. While some of these species were incorporated into the BC matrix by non-specific interactions, others have been specifically bound by fusion to a cellulose-binding domain (CBD) protein^{72–74}. These composite materials are all synthesised *in vitro* using chemical and physical methods, which lack the benefits of biological material assembly. However, recent years have seen increasing efforts to harness the benefits of biological assembly by genetically-engineering BC-producing bacteria (Figure 4a).

Initial efforts to genetically engineer BC-producing bacteria focused on improving the yield of BC. In addition to BC, *G. xylinus* is known to produce a second extracellular polysaccharide, acetan⁷⁵. In an early attempt to increase BC yields, an acetan biosynthesis gene was disrupted in *G. xylinus*⁷⁶ – however, it was found that disrupting acetan biosynthesis in fact reduced BC yield. Other studies used random mutagenesis approaches to isolate high-yielding BC-producing strains⁷⁷. More recently, rational engineering approaches have been used to increase BC production. For instance, knocking out *G. xylinus* glucose dehydrogenase (GDH), which competes with BC biosynthesis for glucose consumption, was shown to boost BC yields⁷⁸. Further, plasmid-based overexpression of the *bcs* operon from a strong, inducible promoter has been shown to increase BC yields from *G. xylinus* by 2-4 fold⁷⁹.

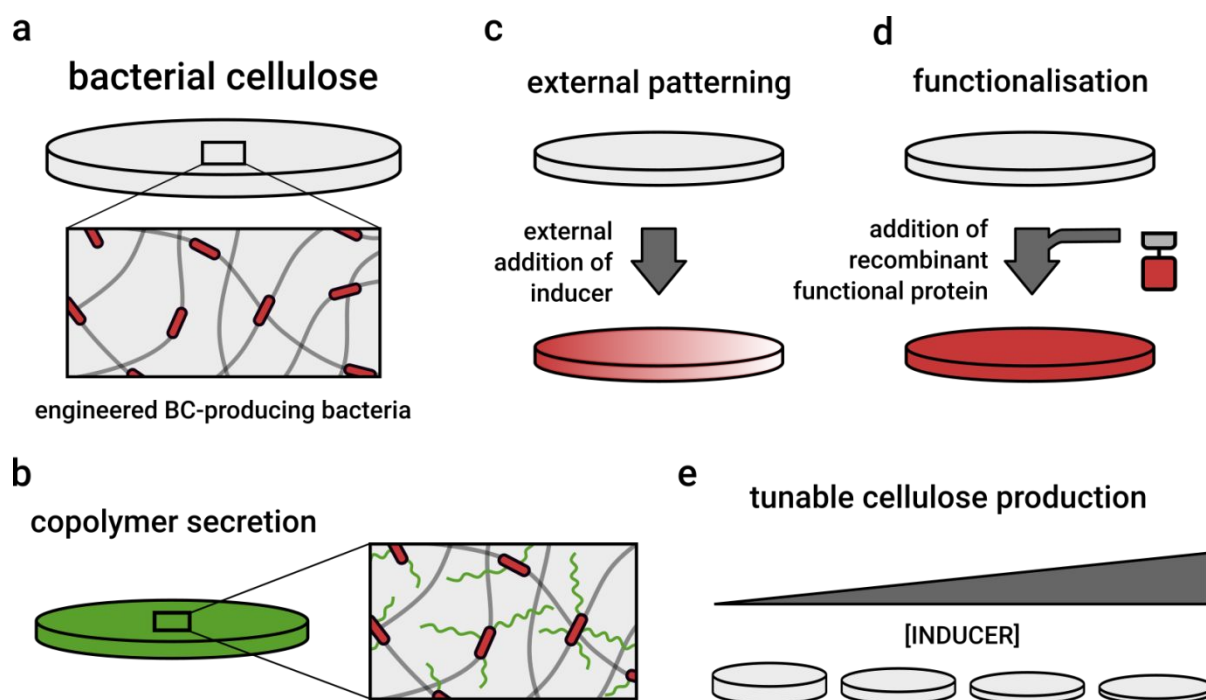


Figure 4 Bacterial cellulose as a biological ELM. (a) BC-producing bacteria can be engineered to produce modified BC materials. A growing number of studies have used this approach to produce growable ELMs with novel properties. (b) Engineering BC-producing bacteria to co-secrete additional polymers, such as curdlan, creates BC composite materials with modified physicochemical properties. (c) Controlling the production of heterologous proteins from BC-producing bacteria using inducible promoters enables external patterning. (d) BC materials can be functionalised *post hoc* by adding functional protein modules. (e) The yield of BC can be tuned by engineering inducible control of BC biosynthesis.

More recently, BC-producing bacteria have been engineered to secrete additional chemical species to create copolymers with modified material properties (Figure 4b). In a landmark study, Yadav et al.⁸⁰ used a metabolic engineering approach to modify the material properties of BC produced by *G. xylinus*, creating a BC-chitin copolymer. Chitin, like cellulose, is a polysaccharide. However, rather than glucose monomers, chitin is made up of chains of N-acetyl-glucosamine (GlcNAc) monomers. The additional acetyl amine group in the sugar units results in the formation of a distinct network of hydrogen-bonds between chitin chains, compared to cellulose. Since the *G. xylinus* cellulose synthase machinery had previously been shown to be able to incorporate UDP-GlcNAc into BC, Yadav et al. hypothesised that producing cytoplasmic UDP-GlcNAc would enable production of a chitin-cellulose copolymer. Indeed, by expressing a *Candida albicans* three-gene operon for UDP-GlcNAc biosynthesis, it was shown that UDP-GlcNAc could be synthesised and incorporated into growing glucan chains. The resultant chitin-cellulose copolymers were shown to have altered material properties: including decreased crystallinity, increased susceptibility to lysozyme degradation and improved *in vivo* degradability. Another study reported engineering of *G. xylinus* to create curdlan-cellulose composites⁸¹. Curdlan is a polymer of β -(1 $\rightarrow$ 3)-linked glucose monomers, noted for its ability to form gels with applications in the food and biomedical industries. The authors engineered *G. xylinus* to express the *crdS* gene from *Agrobacterium tumefaciens*, which naturally produces large quantities of extracellular curdlan. The CrdS protein is predicted to be the major curdlan synthase enzyme and shares homology with the catalytic BcsA subunit of cellulose synthase⁸². It was found that heterologously-expressed CrdS was able to direct curdlan biosynthesis alongside natural BC production, and the resulting pellicles exhibited altered surface morphology and decreased water permeability.

To aid and expand on these efforts, Florea et al. developed and utilised a modular genetic toolkit to modify a newly-isolated BC-producing strain, *Komagataeibacter rhaeticus*⁸³. Compared to other high-producing strains, *K. rhaeticus* was more readily transformed with plasmid DNA. Using the principles of synthetic biology, the authors set out to create a genetic toolkit to facilitate *K. rhaeticus* genetic modification. A panel of modular genetic parts were generated and characterised, including origins of replication, inducible and constitutive promoters, and fluorescent reporter proteins. These genetic tools were then

leveraged to control the spatial and temporal patterning of BC through the application of chemical inducers of gene expression (Figure 4c). Here, *K. rhaeticus* was engineered to produce intracellular fluorescent proteins in response to a chemical inducer. By varying the spatial and temporal application of the inducer, patterned BC materials were created. In addition, as an alternative to *in situ* biological functionalisation, BC was functionalised by adding previously expressed and purified recombinant fluorescent proteins fused to cellulose binding domains (Figure 4d). In another demonstration of the utility of this genetic toolkit, Florea et al. showed that the yields of BC biosynthesis could be externally controlled (Figure 4e). To achieve this, an inducible promoter was used to control the expression of an RNA repressor system. Here, an RNA sequence was designed to target the mRNA encoding the enzyme responsible for UDP-glucose synthesis. Once bound to the target mRNA, the RNA recruits a heterologously-expressed *E. coli* Hfq protein, which brings about inhibition of mRNA expression and therefore shuts down BC biosynthesis. This system was not only highly-effective, enabling arrest of cellulose synthesis at high levels of induction, but also tunable, enabling intermediate levels of suppression at lower levels of induction. More recently, Walker et al. expanded this genetic toolkit, engineering small molecule cell-cell communication in *K. rhaeticus*⁸⁴. Importantly, intercellular communication tools such as these may enable programmable control of BC morphology through pattern formation.

Thanks to its genetic tractability, relative simplicity and desirable natural material properties BC is a useful model biological ELM system. Indeed, this is a growing area of study, and there may be further opportunities to genetically-engineering new BC material properties. Current studies have shown that BC-producing bacteria can be engineered to synthesise curdlan-cellulose and chitin-cellulose copolymers. However, bacteria naturally produce a vast number of other extracellular polysaccharides, many with important industrial uses^{85,86}. Engineering co-secretion of these polysaccharides from BC-producing bacteria could enable the production of a variety of novel copolymer materials. Similarly, numerous reports have demonstrated that purified proteins can be externally added to BC to confer new and useful material properties, for instance, increased hydrophobicity or catalytic activity^{63,87,88}. Going forwards, it may be possible to engineer BC-producing bacteria to secrete proteins themselves and therefore functionalise BC *in situ*. However, compared to standard synthetic biology model organisms, there is a paucity of genetic tools for engineering BC-producing

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bacteria and limited understanding of their cell physiology. For instance, the native mechanisms of regulation of cellulose production remain poorly understood. Similarly, the capacity of BC-producing bacteria to secrete heterologous proteins remains unknown. The continuing development of modular genetic tools and continuing insights into the natural cell physiology of BC-producing bacteria will play a vital role in achieving these goals.

Co-culture approaches for bacterial cellulose biological ELMs

An alternative approach to create BC-based ELMs relies not on engineering BC-producing bacteria themselves, but instead co-culturing BC-producing bacteria with another engineered microorganism. Genetic tools for engineering BC-producing bacteria and detailed molecular characterisation of BC-producing bacteria have only relatively recently been developed. Co-culturing BC-producing bacteria with a model organism for which numerous genetic tools and circuits have been developed may therefore facilitate and accelerate ELM development.

Generally, engineering so-called cellular consortia – co-cultures of two or more microbes – is a topic of increasing interest⁸⁹. Cellular consortia have some broad advantages over mono-cultures, such as improved robustness and the division of labour between individual strains⁹⁰. For example, by distributing the biosynthetic pathway between co-cultured *E. coli* and *S. cerevisiae* production of a precursor of the anticancer drug paclitaxel was enhanced⁹¹. In addition, as exemplified in bacterial biofilm ELMs, engineered cellular consortia have been shown to enable tunable and autonomous patterning of a biological material¹³. Notably, a similar process of specialisation occurs in complex natural biological materials. For instance, in human skin, keratinocytes produce keratin to give mechanical strength, fibroblasts produce collagen and elastin which further strengthen the extracellular matrix, melanocytes produce melanin to confer colour and Langerhans cells detect and respond to the presence of pathogens⁹².

A similar situation can be envisioned to create novel BC ELMs, where BC-producing bacteria create the scaffold BC matrix and an engineered model organism contributes an additional functional property. Using this approach, a recent study manually incorporated engineered *E. coli* into grown BC⁹³. By externally applying *E. coli* cells to the surface of BC part-way through

the process of pellicle formation, *E. coli* cells could be entrapped within the growing BC matrix. The authors showed that engineered *E. coli* could be incorporated into BC and could be chemically-induced to express the reporter protein GFP. In a follow-up study, BC pellicles into which *E. coli* had been incorporated were saturated with a pre-purified biopolymer protein, silk fibroin (SF)⁹⁴. Introduction of SF to BC increased the transparency of the resulting material and reduced the escape of incorporated *E. coli* cells. A more complex *E. coli* genetic circuit was then used – a previously-described dual-colour riboswitch, in which addition of a chemical inducer switches GFP expression off and RFP expression on. The authors showed that this riboswitch system functioned well when incorporated into BC-SF materials, creating dynamic, functional living materials able to sense-and-respond to their environment. In the future, it is hoped these materials can be developed to act as environmental biosensors, sensing and reporting external stimuli. This approach clearly demonstrates that engineered *E. coli*, co-cultured with BC-producing bacteria can endow new functional properties to BC to create novel biological ELMs.

Genetically programming biological ELM morphology

One of the most remarkable properties of natural biological materials is the ability of living cells to control the morphology of the materials they produce. Specifically, living cells execute genetically-programmed rules to autonomously control the morphology of natural biological materials over multiple length scales. In fact, many of the impressive physical properties exhibited by natural biological materials rely on the formation of specific structures across multiple length scales. Inspired by these processes, the field 'synthetic morphogenesis', aims to use tools and techniques from synthetic biology to "engineer, program, grow, and maintain biological systems with complex structures"⁹⁵. One particular aspect of synthetic morphogenesis of great interest for the development of biological ELMs is pattern formation⁹⁶. Pattern formation describes the process by which cells become differentiated spatially. Once patterns have formed, cells follow different gene expression programs and can, as a result, produce patterned biological materials. In this section we will discuss a number of mechanisms for pattern formation, focussing specifically on

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3 autonomous mechanisms, in which cells spontaneously self-organise into specific
4 morphologies. We focus in particular on how these mechanisms have been recreated
5 synthetically in microbial systems and engineered unicellular eukaryotes. Although beyond
6 the scope of this review, the reader is referred to Scholes et al.⁹⁶ and Teague et al.⁹⁵ for
7 excellent, in-depth discussion of the mechanistic basis and future prospects of pattern
8 formation and synthetic morphogenesis.
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17 **The French Flag model**

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19 In the French Flag model, a spatial gradient of an external chemical morphogen alters
20 cellular gene expression to create patterns (Figure 5a). This simple system can be built up
21 across multiple dimensions and over hierarchical levels to create complex patterns and
22 structures. In one of the first examples of a synthetic pattern-forming system, *E. coli* cells
23 were engineered to produce fluorescent proteins in response to different concentrations of
24 the quorum-sensing inducer AHL⁹⁷. Synthetic 'band detector' genetic circuits, were designed
25 to enable transcriptional activation in response to user-defined AHL concentration ranges.
26 When grown on solid media, these band-detector strains formed rings of fluorescence
27 around AHL-synthesising strains⁹⁷. A similar approach was used to demonstrate that multiple
28 morphogens can spatially control gene expression over two- and three-dimensions (Figure
29 5b)⁹⁸. Building on these tools, Grant et al. created orthogonal AHL cell-cell communication
30 systems to create long-range signal relays⁹⁹.
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41 In a recent extension of the basic French Flag approach, Boehm et al. created a three-colour
42 French Flag system enabling hierarchical patterning of gene expression from *E.*
43 *coli*¹⁰⁰. Engineered cells produced different fluorescent proteins in response to different
44 concentrations of two orthogonal AHL inducers, creating a two-colour pattern. But in
45 addition, each AHL inducer controlled the expression of a split RNA polymerase, which in
46 turn activated the expression of a third fluorescent protein. This second genetic circuit acted
47 as an AND logic gate – activating gene expression only in the presence of both AHL inducers
48 – to create a second level of patterning. The authors propose that, by engineering patterned
49 cells to produce additional orthogonal AHL signalling molecules, higher-order patterning can
50 be achieved in the future.
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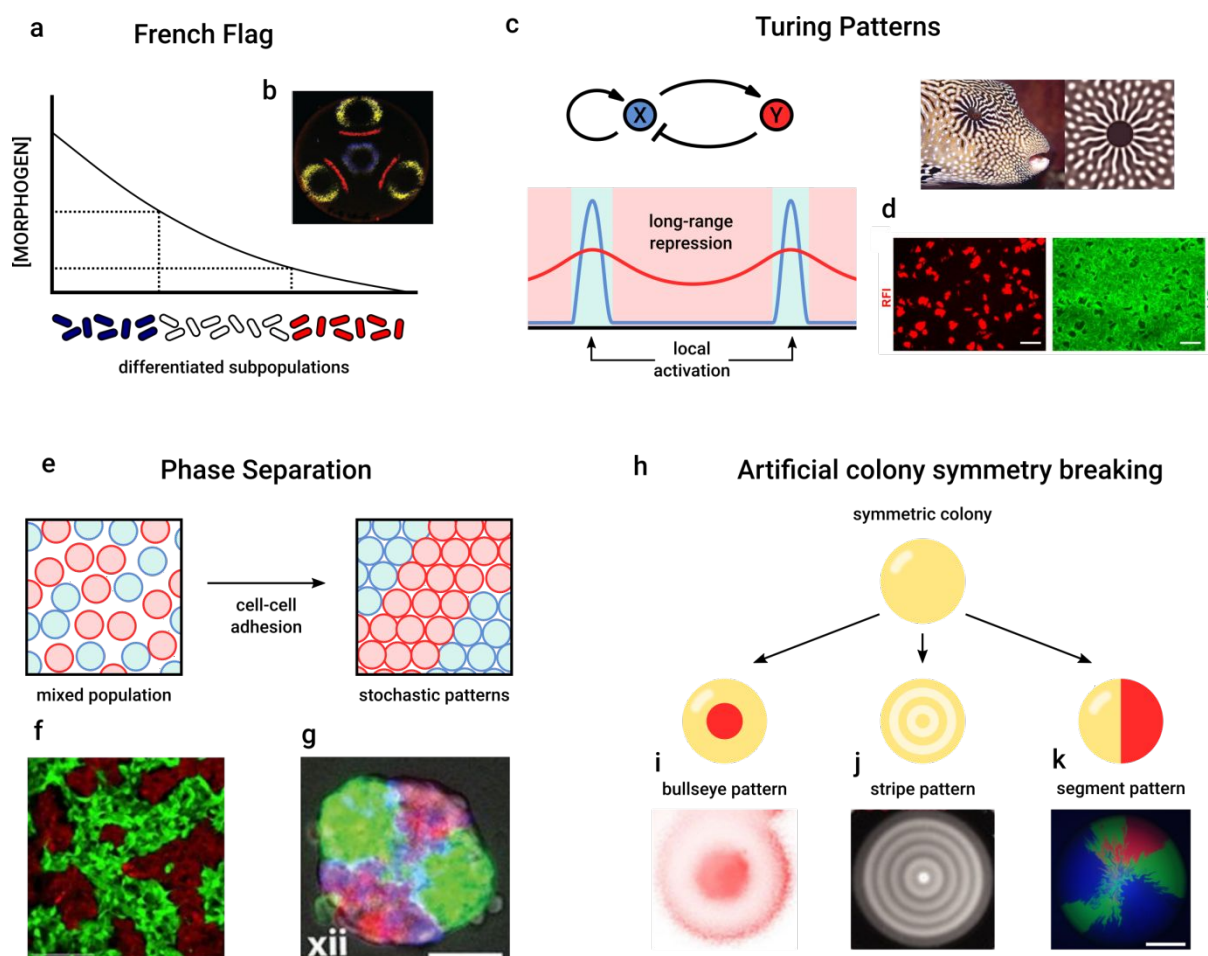


Figure 5 Natural and engineered mechanisms for morphogenetic control. (a) The French Flag system. Here spatial gradients of a morphogen or morphogens induce differentiation of a field of identical cells. (b) Morphogen gradients lead to patterning in a lawn of engineered *E. coli* across two-dimensions, from Sohka et al.⁹⁸ (c) Turing patterns. Turing patterns are established by two species, a slow-diffusing species (X) which activates the production of both itself and a second fast-diffusing species (Y) which represses the production of X. The interaction of these species creates local regions of activation and long-range regions of repression. In natural biological systems, X and Y can act as morphogens, creating complex patterns as seen in popper fish coloration¹⁴¹. (d) Engineered Turing-type pattern formation in a lawn of *E. coli* cells expressing either GFP or RFP, from Karig et al.¹⁰³ (e) Phase separation. Here an initially homogeneously-distributed mixed population is segregated by the propensity of subpopulations to adhere to one another forming stochastic patterns. (f) Patterning of engineered mammalian cells expressing GFP or RFP through phase separation from Cachat et al.¹⁰⁵. (g) Engineering pattern formation in mammalian multicellular aggregates through differential cell-cell adhesion and cell-cell communication, from Toda et al.¹⁰⁸. (h) Engineering symmetry breaking within a growing colony. Starting from an initially homogenous, symmetric colony, various approaches have been developed to create patterned structures. Images are included showing examples of engineered systems for (i) bullseye patterns (from Payne et al.¹¹⁰), (j) stripe patterns (from Liu et al.¹¹¹) and (k) segmentation patterns (from Nunez et al.¹¹³).

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It should be noted that the French Flag system relies on an externally-derived morphogen gradient. Therefore, the systems described here do not strictly result in autonomous patterning. Indeed, even in nature, French Flag patterning mechanisms are initiated externally. For instance, during *Drosophila* embryo development, maternally-produced morphogen proteins create the anterior-posterior axis, although higher levels of patterning are then generated autonomously within the embryo.

Turing patterns

Turing patterns arise in natural biological systems based on the reaction and diffusion of two morphogen species: a slow-diffusing species that activates the production of itself and of the other species, and a fast-diffusing species that represses the production of the activator (Figure 5c). As a result, local increases in the levels of the activator are self-reinforcing, while simultaneously creating surrounding regions of repression.

There have been numerous efforts to use the wealth of synthetic biology genetic tools for cell-cell communication to engineer Turing patterns, yet so far these have met with limited success. So much so, that Turing patterns have been given the tongue-in-cheek moniker “the graveyard of synthetic biology”⁹⁶! The basis of this difficulty seems to be the narrow range of parameters that must be satisfied to give rise to Turing patterns, particularly with respect to the difference in diffusion rates of the activator and the repressor. Many of the current cell-cell communication systems available to synthetic biologists rely on small molecules – peptides and AHLs¹⁰¹, for example – whose diffusion rates differ only minimally. Borek et al. proposed the use of activating AHLs in combination with gaseous hydrogen peroxide (H₂O₂) as a fast-diffusing, long-range inhibitor¹⁰². Alternatively, it may be possible to physically restrict the diffusivity of a cell-cell signalling species by tethering to the cell surface or by engineering it to bind an underlying material scaffold.

Excitingly, two recent reports have described engineered pattern formation through reaction-diffusion mechanisms in *E. coli* and in mammalian cells. In the first of these studies, pattern formation was engineered in *E. coli* using a non-classical, stochastic Turing-type mechanism¹⁰³. In an expansion of Turing’s original theory, stochastic Turing theory includes noisy activator-inhibitor birth and death processes. One consequence of this stochastic

theory is a broader range of parameters over which patterns can emerge, including the required difference between the diffusion rates of the activator and inhibitor. Using two orthogonal bacterial quorum-sensing systems, the authors engineered such a stochastic activator-inhibitor genetic circuit, producing patterns within a lawn of initially homogeneous cells (Figure 5d).

In the second study, the Nodal-Lefty cell-cell signalling pathway was used to create patterns in mammalian cells based on a reaction-diffusion mechanism¹⁰⁴. The signalling molecules, Nodal and Lefty, had previously been shown to exhibit the required properties for reaction-diffusion patterning: Nodal is a short-range activator that activates its own production and that of Lefty, while Lefty is a long-range inhibitor of Nodal. Here, Sekine et al. showed that Nodal and Lefty can give rise to the spontaneous formation of patterns in engineered HEK293 cells. Importantly, the authors note that two possible reaction-diffusion type mechanisms could account for their observed patterns, a true Turing pattern or a stable 'solitary pattern' mechanism. Regardless, the study demonstrates that Nodal-Lefty signalling enables programmable pattern formation based on a reaction-diffusion mechanism.

These landmark studies have implications both for the further development of Turing-type patterns and for the understanding of mechanisms of natural pattern formation. Despite their complexity, since Turing patterns are truly autonomous and can direct formation of a variety of structures – stripes, spots, labyrinths – efforts to engineer them are of great value for development of biological ELMs and are likely to continue.

Phase separation

The phase separation model creates self-assembled patterns of cells based on differential adhesion between cellular subtypes (Figure 5e). Analogously to the separation of oil and water, cell subtypes that exhibit different propensities for adhesion to one another will become spatially-segregated. If the movement of cells is constrained, incomplete segregation will result in the formation of clusters of each subtype, creating a patterned system. Cachat et al.¹⁰⁵ used the phase separation model to engineer autonomous patterning of mammalian cells. Mammalian cells were engineered to express cadherins – calcium-dependent cell surface adhesion proteins – enabling specific pairwise cell-cell adhesion¹⁰⁶.

Two self-adhering cell lines were engineered, one expressing GFP and one expressing RFP. When grown in mixed cultures, these cell lines spontaneously sorted into intricate two- and three-dimensional patterns, the properties of which could be tuned simply by modifying the proportions of the two cell types (Figure 5f). More recently, a modular genetic toolkit was developed for programming cell-cell adhesion in *E. coli* through the surface display of a library of protein binding pairs¹⁰⁷. Glass et al. used this system to engineer adhesive selectivity between cell subtypes, generating multicellular aggregates of *E. coli* able to self-assemble into a number of patterns and morphologies.

In a remarkable recent extension of this approach, differential cell-cell adhesion was coupled to cell-cell signalling to program self-organising multicellular structures¹⁰⁸. Here, cell-cell communication was engineered into mouse L929 fibroblasts using the synNotch receptor system. Through the interaction of cell surface-displayed receptors and ligands, the synNotch system enables communication between physically-associated sender and receiver cells¹⁰⁹. The authors showed that, by mixing multiple cell types in which synNotch signalling induced the expression of specific combinations of cadherins, cells could self-organise into two- and three-layer spheroid structures (Figure 5g). One disadvantage of this approach is the requirement for prior mixing of different cell types. To meet this limitation, the authors employed an autonomous cell fate bifurcation system, allowing a single genotype to spontaneously differentiate into two subpopulations which could then self-organise into multicellular structures. Further, although exemplified with relatively small spheroid structures, theoretically, this approach could be applied to increasingly large scale multicellular systems.

While phase separation systems enable fully autonomous formation of patterns with consistent general properties, it should be noted that the exact morphologies of patterns cannot be precisely predicted and are unique on each occasion. However, owing to the relative simplicity of the phase separation model for pattern formation, future years may well see its use to create growable biological ELMs with genetically-programmed patterns.

Artificial colony symmetry breaking

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3 A number of alternative approaches have been described to rationally engineer the
4 morphology of individual, growing *E. coli* colonies. While these strategies employ a diversity
5 of different underlying mechanisms, they share a common goal: creating spontaneous
6 patterns of differentiated cells starting from single cells (Figure 5h). One approach uses the
7 accumulation of an AHL cell-cell communication molecule essentially as a timer, triggering
8 cell-differentiation at a certain stage of colony growth when AHL concentration breaches a
9 particular threshold. Using this general principle, Payne et al.¹¹⁰ created a genetic switch, in
10 which a threshold level of AHL results switches off one promoter and switches on another.
11 Consequently, during the growth of single colonies, AHL levels accumulate and eventually
12 flip this genetic switch, creating self-organising bullseye ring patterns (Figure 5i). In a
13 remarkable extension of this approach, Liu et al.¹¹¹ created a genetic circuit in which AHL
14 accumulation leads to suppression of *E. coli* cell motility. Based on the complex interplay of
15 the dynamics of AHL concentration, cell density and nutrient availability, the engineered cells
16 spontaneously self-assemble into stripes of alternating high and low cell density regions
17 (Figure 5j). In addition, the authors demonstrate that the spacing of stripes could be
18 controlled by fine-tuning the expression of a single gene involved in motility.

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20 Rudge et al.¹¹² highlighted the role of the mechanical and geometric properties of cells in
21 creating self-organising patterns. Tracking the interfaces between individual subpopulations
22 in growing bacterial colonies revealed the spontaneous emergence of jagged, fractal
23 patterns. Using mathematical modelling, the authors showed that these patterns were
24 generated by polar cell shape and end-to-end cell division.

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26 In the same study, Rudge et al. described a system allowing spontaneous differentiation of
27 cellular subpopulations within a symmetrical colony. Here, *E. coli* cells were transformed with
28 two plasmids harbouring the same origin of replication, each encoding resistance to two
29 antibiotics, one shared, one unique. When grown in media selecting for the presence of the
30 two unique resistance markers, cells harboured both plasmids. However, when plated onto
31 solid agar selecting for the presence of the single, shared antibiotic resistance marker, cells
32 could spontaneously rid themselves of one or other plasmid. This process results in
33 segregation of the two plasmids early on in colony formation and, therefore, breaks the
34 colony symmetry creating segments of genetically-differentiated subpopulations. In a follow-
35 up study, the number of subpopulations was expanded by increasing the number of co-

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transformed plasmids¹¹³. This symmetry-breaking system could then be used to engineer domain-specific gene regulation, enabling spatial patterning of colony morphology and of metabolite production (Figure 5k).

Biological ELM systems of the future

While current efforts to develop biological ELMs have focussed on simple microbial systems such as bacterial cellulose and *E. coli* biofilms, in the coming years more sophisticated and flexible systems are likely to emerge. In this section we discuss two general strategies that can be envisioned for the development of more complex biological ELM systems: targeting increasingly complex natural biological materials and building and patterning novel materials from the bottom up.

Engineering complex natural biological materials

The most sophisticated early examples of biological ELMs – bacterial cellulose and *E. coli* biofilms – share a common general approach. Specifically, starting from naturally-occurring biological materials, researchers have engineered novel properties. Going forwards, attention may turn to more complex natural biological systems – marine molluscs could be engineered to produce modified mineralised materials, trees could be engineered to produce wood with modified properties. If successful, such an approach would allow researchers to select natural biological materials exhibiting basic desirable properties on top of which new, useful properties could be engineered. Indeed, recent years have seen more and more instances of genetic engineering of increasingly complex organisms for biological material production. Simple biomineralised structures, such as magnetosomes and diatom silica cell walls have been engineered to produce functional nanomaterials^{114–120}. The silkworm, *Bombyx mori*, has been engineered to produce modified silk incorporating heterologous proteins and unnatural amino acids^{121–124}. And genetic tools have been developed that could be used to engineer natural composite materials produced by fungal mycelia^{125–127}.

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3 However, attempting to engineer complex and multicellular systems brings with it a whole
4 new set of challenges. The long generation times of hosts such as plants and trees precludes
5 fast cycling of the design-build-test-learn process. Further, the properties of natural
6 biological systems are dictated by the particular evolutionary selective pressures experienced
7 by the native hosts. As such, their biological and physical properties are, to an extent,
8 predetermined and inflexible for downstream modification. This latter limitation is true even
9 for the simple, microbial biological material systems described above. For example, the
10 biosynthesis of bacterial cellulose is highly-regulated in the native host by mechanisms that
11 are not currently fully-understood, while *E. coli* is a relatively poor host for the secretion of
12 heterologous proteins with which curli fibres could be functionalised.
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24 **Building novel biological ELMs from the bottom up**

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26 An alternative strategy to the development of future biological ELMs might instead focus on
27 engineering novel, non-natural materials from the bottom up. To achieve this, model
28 microbial organisms that are tractable with standard synthetic biology tools would be
29 rationally engineered to self-assemble designed materials with user-defined properties.
30 Engineering biological material formation *de novo* in a model organism would offer much
31 greater, user-defined control over the biological ELM material properties. In fact, a broad
32 framework for such an approach using protein building blocks was outlined recently². In this
33 approach, microbes are engineered to express and secrete multiple proteins into the culture
34 medium. Secretion of self-assembling structural proteins – such as CsgA or elastin-like
35 polypeptides (ELPs) – could enable the formation of a polymeric scaffold. Simultaneously,
36 functional protein modules – such as enzymes, adhesion domains or mineralisation peptides
37 – could be secreted and conjugated to the structural protein, creating a functionalised
38 material. Owing to the modular nature of protein domains, the specific properties of the
39 resultant material could be user-defined by picking and choosing the desired structural and
40 functional protein modules. Given the seemingly endless variety in natural structural and
41 functional protein domains, a huge diversity of material properties could, in theory, be
42 achieved using this approach.
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58 One potential embodiment of this system would be to co-culture multiple strains, each
59 engineered to secrete different protein modules. Firstly, this would prevent intracellular
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3 conjugation of co-expressed functional and structural proteins. But in addition, a co-culture
4 system could facilitate efforts to genetically-program biological ELM morphogenesis. By
5 using pattern-forming genetic circuits, gene expression from different strains could be
6 differentially regulated, both spatially and temporally. In fact, as above, this same approach
7 was used by Chen et al. to control the pattern of CsgA monomers along curli fibres¹³.
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12 Despite offering substantial potential advantages, particularly over engineering naturally-
13 occurring biological materials, developing such a system will require a number of major
14 challenges to be met. Chief amongst these will be efficient secretion of a range of
15 recombinant proteins, as well as methods for stable and specific conjugation of secreted
16 proteins. Several recent reports have aimed to meet these challenges. Azam et al. described
17 a general strategy for secretion of biopolymer-forming proteins using the *Salmonella*
18 *enterica* type III secretion system¹²⁸. Further, unnatural amino acid incorporation and the
19 previously-mentioned SpyTag-SpyCatcher system have been used to conjugate recombinant
20 proteins *in vitro* to create self-assembling protein materials^{129–132}. Combining these processes
21 of protein secretion and conjugation, we recently described how *B. subtilis* can be
22 engineered to secrete proteins which subsequently become covalently conjugated through
23 the SpyTag-SpyCatcher reaction in the extracellular environment¹³³. Crucially, however, the
24 approaches used to secrete heterologous recombinant proteins remain limited to milligram
25 per litre yields, restricting the scalability of material production. While these reports
26 represent early steps towards bottom up design of biological ELMs, many challenges remain.
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43 Outlook

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45 Going forwards, it is likely that a combination of top down and bottom up approaches will
46 be most productive. For instance, host organisms producing natural biological materials
47 could be re-engineered to remove restrictions imposed by their natural characteristics. For
48 instance, approaches such as pathway refactoring^{134–136} could be used to remove native
49 regulation of material production or non-native functions could be installed, such as
50 improved ability to secrete heterologous proteins. Similarly, the development of *de novo*
51 biological ELMs might be accelerated by looking to natural biological materials for
52 inspiration.
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3 In addition, the development of biological ELMs for real-world applications will require
4 further challenges to be met. In particular, cost-effective production of materials at large
5 scale may limit the viability of biological ELMs for specific applications. For instance, despite
6 methods to improve production scalability, the yields of curli fibre materials remain restricted
7 to the milligram per litre scale¹³⁷. Similarly, the yields of proteins that can be secreted from
8 microbial hosts, particularly the yields of heterologous proteins, are often limited to tens of
9 milligrams per litre^{138,139}. Consequently, the field of biological ELMs may see a shift in focus
10 to material systems that naturally enable the efficient production of larger quantities of
11 material. Yields of BC, for instance, can reach in excess of 10 grams per litre. This discrepancy
12 in yields may reflect the fact that the production of carbohydrate biopolymers generally
13 consumes significantly less energy than that of protein biopolymers. Alternatively,
14 production yields may be improved in material systems in which inorganic species or the
15 entire cell mass forms a major part of the material, such as biomineralised materials or fungal
16 mycelium, respectively. Finally, it is worth noting that many of the advantageous properties
17 of biological ELMs rely on the presence of viable, genetically-engineered cells within these
18 materials. This raises questions not only from a practical point of view – such as how to
19 maintain living cells in a viable state within materials – but also regarding how biological
20 ELMs might fit into public opinion of genetic engineering and regulatory frameworks.
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Summary

Here, we have explored current and emerging systems for the development of genetically-programmable functional materials – biological ELMs. This field aims to take advantage of the impressive properties of natural biological materials, such as autonomous patterning, diverse chemical and physical properties and the ability to sense and respond to the environment. Achieving this bold aim will be a major challenge, requiring the rules governing the formation and function of natural biological materials to be discerned and re-engineered into complex synthetic genetic circuits. To date, the complexity of biological ELM systems that we can rationally engineer remains vastly inferior to that which has arisen in natural biological materials.

However, if realised, this vision could enable a paradigm shift in the production of materials, leading to a future where materials with completely novel properties can be grown by microbes, rather than manufactured. Non-biodegradable materials and unsustainable manufacturing processes could be replaced by biodegradable materials that self-assemble under mild conditions, fed by sustainable raw materials and energy sources. Living construction materials and textiles could be endowed with the ability to self-repair after damage. Wastewater treatment could be carried out by living materials able to simultaneously screen for and report the presence of toxins and pollutants. Examples like these may seem far-fetched, but when we consider the complexity of natural biological materials, it is clear that the limiting factor in these efforts is not the innate capacity of biology, but instead our ability to rationally engineer these systems. Whether these goals can ever be achieved remains an open question, but this growing field is taking the first steps towards answering those questions.

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