Accessing biology's toolbox for the mesoscale biofabrication of soft matter


Biology is a master of mesoscale science, possessing unprecedented capabilities for fabricating components with nano-scale precision and then assembling them over a hierarchy of length scales. Biology's fabrication prowess is well-recognized and there has been considerable effort to mimic these capabilities to create materials with diverse and multiple functions. In this review, we pose the question – why mimic, why not directly use the materials and mechanisms that biology provides to biofabricate functional materials? This question seems especially relevant when considering that many of the envisioned applications – from regenerative medicine to bioelectronics – involve biology. Here, we provide a sampling to illustrate how self-assembly, enzymatic-assembly and the emerging tools of modern biology can be enlisted to create functional soft matter. We envision that biofabrication will provide a biocompatible approach to mesoscale science and yield products that are safe, sustainable and potentially even edible.

The potential for biology to contribute to materials science is increasingly being recognized. Recently, several in-depth reviews have appeared on materials based on nucleic acids, proteins, and polysaccharides. In addition to the materials for construction, biology also provides the mechanisms for construction that enable assembly over a hierarchy of length scales. These mechanisms include the templated biosynthesis to create structure at the nanoscale (e.g., proteins), as well as the methods to connect these nanoscale components by self-assembly and enzymatic-assembly. In this brief review, we emphasize the breadth of biofabrication which we define as building with biological materials and mechanisms. In addition, we use a series of examples to illustrate the potential of biofabrication, especially self-assembly and enzymatic-assembly, to address the challenges of mesoscale science.

Background

At a very broad level, it is interesting to consider the materials science differences between biology and technology. Table 1 highlights some of the unique biological approaches at three different levels, the strategic (how to “design?”), the tactical (how to build?) and the results (what are the features of the product?).

Biology’s “design” strategy

To a large extent, biology builds high-performance materials from soft matter (e.g., biopolymers). If we could access biology's soft matter fabrication capabilities we could significantly
complement existing technological capabilities for fabricating hard materials (e.g., microelectronic chips). In addition, biology builds in aqueous solutions and creates systems that function in water; again complementary to many technological methods and products derived either from organic chemistry or device microfabrication.

A critical challenge to mesoscale science is the amount of information needed to effectively design materials over the length scales that vary from the molecular to the macroscopic. As suggested in Table 1 biology sidesteps this challenge by employing a discovery-based approach to “design”. For instance, natural variability coupled with selective pressure enable the evolution of species over long time frames while evolution over shorter time frames allows viruses (e.g., HIV) to acquire resistance. Often variability and selection are considered to be uncontrollable activities, but selection is quite purposeful when the adaptive immune system creates an antibody against a new pathogenic threat. Analogous purposeful approaches to “direct-evolution” have been developed by biotechnologists to enlist biology’s discovery-based learning approach to generate functional materials (e.g., binding peptides) in the absence of the complete knowledge required for de novo design. Biology also uses modularity for materials synthesis. Modularity is illustrated by proteins, where sub-nano-scale building blocks (e.g., amino acids) are assembled into polypeptides where short sequences can form secondary structural elements (e.g., α-helices) that further associate to form fully folded nano-scale tertiary structures. The folded protein may have more than a single functional domain (e.g., separate domains may exist for binding and catalysis) and this folded protein may associate with other proteins or membranes to form larger functional units (e.g., the electron transport chain in mitochondria).

### Biology’s fabrication tactics

In addition to its complementary approaches to design, Table 1 shows that biology offers unique methods for mesoscale construction. Through selective catalysts (i.e., enzymes) biology accesses chemicals from its environment and either converts the chemical energy into a usable form or rearranges the elements (e.g., the carbon skeleton) into intermediates and products that enable life. Enzymes are appealing technological catalysts because of their selectivity, their functioning under mild aqueous conditions, and their ability to be engineered and produced through biotechnological methods. Enzymes have been widely studied for applications in foods, fuels, and chemicals; we believe there remains considerable opportunity for applying the power of biocatalysis for materials synthesis.

One hall-mark of biological soft-matter fabrication is the precision manufacturing of macromolecules through templated biosynthesis. In this case, the biosynthetic information is stored as modular units (DNA sequences) while high-fidelity processes of transcription and translation generate proteins of precise sequence and size that fold into a single native structure with a precise spatial orientation of the individual amino acid residues. The nano-scale precision enabled by templated biosynthesis is difficult (or impossible) to achieve through alternative synthetic routes. Biology also uses non-templated biosynthetic mechanisms to generate lower-fidelity biopolymers (e.g., polysaccharides).

In addition to templated biosynthesis, another hall-mark of biological fabrication is its use of non-covalent bonds for the bottom-up self-assembly of supra-molecular structure. Many biopolymers, whether extracted from natural sources or generated in biotechnological processes, possess such self-assembling capabilities. While these self-assembling capabilities are well-appreciated for traditional food (gelatin gels) and life science applications (agarose gels), we believe the biopolymer self-assembling properties provide exciting opportunities for newer “high-tech” applications. We cite several examples in later sections to illustrate the possibilities.

A final hall-mark of biological fabrication is the use of molecular recognition. While molecular recognition is well-known for biological binding (antibody binding and DNA hybridization) and catalysis (enzyme catalysis), molecular recognition is often used for assembly (virus particle assembly).

### Biology’s resulting soft assemblies

Table 1 shows that in addition to design and fabrication, biology’s soft matter “products” offer several capabilities that have inspired technological efforts. Biological soft materials can respond to their environment either reversibly (e.g., allosteric enzymes) or irreversibly (e.g., the blood coagulation cascade), they can be resorbed, and they can heal. Biology also employs assemblies (e.g., organelles) to create localized microenvironments, to segregate components and compartmentalize functions. And biological soft-systems perform remarkable feats.
such as the harnessing of solar and chemical energy by transduction mechanisms that do not require heat as an intermediate form of energy. Thus biological soft materials offer diverse and enviable capabilities.

In the following, we cite several examples, primarily from our groups’ work, to illustrate the diverse possibilities of biofabricating functional soft matter. Our goal is to provoke a broader appreciation and interest in the use of biology’s materials and mechanisms for mesoscale science.

Examples

Self-assembly

As mentioned, biology routinely employs bottom-up self-assembly to build structure. In this case, information contained in the molecules themselves guide the spontaneous formation of the weak, non-covalent interactions that generates structure. The classic example of biological self-assembly among different molecules is the formation of bilayer membranes from lipids and proteins. Protein-folding is also a self-assembling process in which associations between different regions of a single polypeptide chain leads to the formation of the compact native structure. Because self-assembly relies on interactions that are weak (relative to thermal energy) a large number of such interactions often must act co-operatively to drive the overall assembly process. Further, the weak nature of the interactions suggests that the self-assembly processes can be reversible and the resulting structures are capable of “breathing”, healing and dis-assembling.

Fig. 1 illustrates recent work in which hydrophobic interactions have been exploited for hemostasis. In this case, the aminopolysaccharide chitosan (shown as blue chains) was chemically modified with hydrophobic \( \alpha \)-alkyl moieties (shown in purple). When this hydrophobically modified chitosan (hm-chitosan) was combined with blood cells, the hydrophobes became embedded within the cell membranes, as illustrated by the inset in Fig. 1a. Accordingly, the blood cells were interconnected by the polymer chains into a three-dimensional gel network that supported its own weight, as shown by the inverted tube in Fig. 1a. In contrast, the unmodified chitosan (without hydrophobes) did not connect the cells or form a gel. The ability of the hm-chitosan to gel blood suggests its potential as a hemostatic agent to arrest bleeding from serious wounds. A unique property of the hm-chitosan gel or “clot” is that it can be reversed because it is based on weak, physical associations. Such a reversal induced by a sugar-based supramolecule, \( \alpha \)-cycloextrin (\( \alpha \)-CD) is illustrated in Fig. 1b. The hydrophobic moieties on hm-chitosan chains detached from the blood cells and were sequestered within the hydrophobic binding pockets of \( \alpha \)-CD molecules. In turn, the blood gel was liquefied and it flowed readily in the inverted tube.

In addition to hydrophobic interactions, electrostatic interactions are prevalent in biology. Since many biopolymers are charged, it is straightforward to use these to generate polymeric membranes, beads, and capsules by electrostatic complexation with oppositely-charged species (multivalent salts, surfactants, or other polymers). For example, capsules can be formed by contacting droplets of the cationic biopolymer, chitosan with an anionic biopolymer such as sodium alginate or gellan gum. Fig. 2 shows the structure of the shell in such a capsule. Capsule size can be tuned over a range extending from a few microns to several millimeters using either microfluidic techniques or gas-driven microencapsulation devices. The shell (membrane) thickness can be independently controlled by varying the contact or incubation time of the oppositely charged species.

Fig. 1 Network self-assembly through hydrophobic interactions. (a) Schematic of a blood gel induced by hm-chitosan: hydrophobes from hm-chitosan chains are inserted into cell membranes and the cells are thus connected into a network. A photograph of gelled blood in an inverted tube is also shown. (b) Schematic for the reversal of gelation induced by \( \alpha \)-cycloextrin (\( \alpha \)-CD): the hydrophobes are disengaged from cell membranes and instead sequestered within the hydrophobic cavities of the \( \alpha \)-CD. A photograph of the liquefied sample is also shown.

Fig. 2 Microcapsule formation due to electrostatic attractions between cationic and anionic biopolymers. The capsule is enclosed by a robust shell generated by electrostatic complexation of chains from the two biopolymers. Capsule formation and encapsulation of payload within the capsule are done simultaneously in a single step. By appropriate choice of payload, we can impart different functions to the capsule. Three payloads are illustrated: (i) magnetic nanoparticles (ii) fusion proteins and (iii) nano-scale vesicles or liposomes.
Capsules provide a simple means to compartmentalize structures and functions in ways that mimic a eukaryotic cell. In a cell, the various compartments (organelles) are enclosed by lipid bilayer membranes, which are a few nanometers thick. The membranes enclosing capsules are typically much thicker (several μm) and are generally much more robust and stable (e.g., these membranes will not get disrupted upon a change in solution pH or ionic strength). Capsule membranes are usually semi-permeable: they allow small molecules to pass through, but large macromolecules or nanoscale structures remain entrapped in the capsule lumen. Fig. 2 illustrates that biopolymer capsules can be made to encapsulate various entities, including (i) magnetic nanoparticles to confer magnetic responsiveness to the capsules; (ii) fusion proteins to impart biosynthetic activities to the capsule; and (iii) nanoscale vesicles to confer colorimetric response of the capsules to pH and temperature.

**Triggered self-assembly**

As mentioned, electrostatic interactions are integral to biological self-assembly and often they involve the charged residues of biomacromolecules. For proteins and polysaccharides, electrostatic interactions are typically mediated by weakly acidic (i.e., carboxylate) and weakly basic (i.e., primary amines) substituents such that the balance between attractive and repulsive interactions is quite sensitive to pH. Similarly, the balance between hydrophilic and hydrophobic interactions is dependent on temperature. The important point is that many biological macromolecules are stimuli-responsive and poised to undergo profound changes upon relatively modest changes in conditions (pH, temperature or salt). Familiar examples of biopolymers that couple stimuli-responsiveness with self-assembling properties (i.e., film formation) are gelatin and agarose that are thermally-responsive, alginate and pectin that are Ca^{2+}-responsive, and chitosan that is pH-responsive. We are examining how stimuli-responsive film-forming properties can be used to trigger hydrogel formation at electrode addresses in response to imposed electrical inputs.

Chitosan was the first polysaccharide to be electrodeposited by the neutralization mechanism illustrated in Fig. 3a. In this case, the cathodic electrolysis of water creates the localized high pH conditions that de-protonates chitosan’s primary amines (pK_a ≈ 6.3) and induces a sol–gel transition at the electrode surface. Once deposited, the hydrogel film is stable in the absence of an applied potential provided the pH is retained above about 6.3; the film can re-dissolve under acid conditions. Importantly, chitosan’s electrodeposition is spatially-selective with the film thickness controlled by the deposition time and the film’s lateral dimensions controlled by the electrode’s pattern or by the use of a template. Chitosan’s electrodeposition is versatile and can be coupled with other assembly approaches as illustrated in Fig. 3b which shows the coupling of electrodeposition with layer-by-layer assembly. Electrodeposition confers the spatial control to the formation of the first chitosan layer while sequential contacting with the anionic
polysaccharide alginate and chitosan allows for the controlled growth of multiple polymeric bilayers as illustrated by the microbalance measurements in Fig. 3b. Electrodeposition also localizes assembly to an electrode surface which is especially convenient for electrochemical biosensing applications. This is illustrated in Fig. 3c which shows results from an experiment in which the model biosensing enzyme glucose oxidase (GOx) was assembled at an electrode address within a microfluidic channel. GOx was incorporated into the film by mixing it into the alginate solution used for LbL assembly. The trace in Fig. 3c illustrates the film’s ability to detect and quantify the glucose level in the flowing stream. Finally, it is important to note that electrodeposited polysaccharide matrices may also provide a favorable environment for the long term preservation of biological function (e.g., GOx activity).

The anionic polysaccharide alginate can also be electrodeposited, by an anodic neutralization mechanism, or by Fe3+ (ref. 43 and 44) or Ca2+ (ref. 45 and 46) induced gelation mechanisms. This latter mechanism is illustrated in Fig. 4a which shows that anodic electrolysis of water generates protons that serve to solubilize CaCO3 particles from the deposition solution and locally generate free Ca2+ ions. This localized Ca2+-generation triggers the gelation of the Ca2+-responsive alginate hydrogel. Importantly, Ca2+-alginate deposition can be performed under sufficiently mild conditions to allow the co-deposition of viable prokaryotic and eukaryotic cells. For instance, Fig. 4b shows results from an experiment in which a population of recombinant E. coli was co-deposited with alginate at an electrode address within a microfluidic channel. After deposition, the chemical inducer was introduced into the channel and the entrapped bacteria responded by expressing the marker protein blue fluorescent protein (BFP) as illustrated by the image and plot shown in Fig. 4b. Fig. 4c indicates that electrodeposition can be performed sequentially to generate a model biofilm with stratified bacterial populations; in this case with three different populations of E. coli strains each expressing a different fluorescent protein.

To date, the biopolymer electrodeposition mechanisms currently known rely on charged polymers that are triggered to undergo gel formation in response to changes in pH or ions (e.g., Ca2+). Fig. 5a illustrates that co-deposition allows electrodeposition to be extended to the thermally-responsive and neutral polysaccharide agarose. In this case the deposition solution contains agarose and a comparatively smaller amount of alginate (1 and 0.2 w/v %, respectively) along with CaCO3. This deposition solution must be retained under warm conditions (≈37 °C) to ensure agarose remains in its soluble state. Alginate’s anodic deposition (by the mechanism of Fig. 4a) traps agarose chains while cooling the deposit allows agarose to form its thermally-responsive gel network. The important features of co-deposition are; it can be performed under mild, biologically-relevant conditions, and the dual gel network possesses less charge than alginate. These features are illustrated in Fig. 5b which schematically illustrates experiments in which yeast cells co-deposited within an agarose/alginate network were cultured and then their surface-displayed proteins were immunonanalyzed. Equivalent cells cultured in alginate networks could not be immunoanalyzed because the large anionic antibody molecules could not penetrate the alginate network (presumably due to electrostatic repulsions).
In summary, recent research is demonstrating that convenient electrical signals can be enlisted to tune polymer structure at the macromolecular and microscopic levels. Further, electric inputs provide a means to adjust intermolecular interactions and guide the formation of films and membranes. Here, we highlighted work with stimuli-responsive film-forming biopolymers in which electrical inputs provide the trigger for localized self-assembly (i.e., electrodeposition). For the mechanisms discussed, the deposited films are physical gels with reversible network junctions and the film's structure and properties can be controlled by deposition conditions (e.g., polymer and salt concentrations). From a practical standpoint, electrodeposition is simple, rapid and reagentless and biopolymer electrodeposition is being extensively studied as a means for generating biocompatible composite coatings (e.g., for implants) and for incorporating biological components into microfabricated sensors and devices. While we focused our discussion on the use of electrical inputs to trigger self-assembly (i.e., by electrodeposition), microfluidic contacting can also provide the appropriate triggers for forming particles, capsules and membranes from stimuli-responsive film-forming biopolymers.

Molecular-recognition based self-assembly

Molecular recognition is conferred to self-assembly when weak and complementary interactions are spatially arranged (i.e., pre-organized) to promote binding between the desired partners while discouraging binding of competitors through less favorable binding energies. Biomacromolecules extensively use the discriminating ability of molecular recognition to guide biological self-assembly and these capabilities are increasingly being investigated for technological applications. For instance, the sequence-specific base-pairing of DNA is well-understood and provides a powerful means to control structure through precise molecular interactions. In particular, DNA macromolecules are being created to programmably assemble in two and three-dimensions through processes often referred to as DNA origami. In addition, protein-based structures (e.g., viruses) are being investigated as self-assembling nano-scale templates. Importantly, routine biotechnological methods can be used to precisely alter the surface properties of these protein-based templates through changes in the amino acid sequence (e.g., of the coat protein).

The capabilities of viral templates are illustrated by work with Tobacco Mosaic Virus (TMV). Fig. 6a shows that TMV is assembled from multiple copies of a single coat protein that self-associates with the viral RNA to produce a defined 18 nm x 300 nm rod-shaped particle. Using the known three-dimensional structure of TMV, we genetically modified this structure...
through the insertion of a cysteine codon within the N-terminus of the coat protein open reading frame. As illustrated by the inset in Fig. 6a, the 1cys mutation is recessed within a groove and partially hidden by the C-terminal arm of the coat protein (CP) and thus this residue is not fully exposed. This recessing predictably precludes the cysteine residues on the lateral wall of the virus from forming direct contacts with surfaces. In contrast, the cysteine derived thiol at the end of the virus rod is sufficiently exposed to allow direct contact with the surface as illustrated in Fig. 6b. Thus, the localized nano-environment of the cysteine residue functions to regulate thiol-mediated surface assembly so the TMV rods are assembled in a vertical orientation onto surfaces that include gold, stainless steel, Teflon, SU-8, Si and SiN.84-87 Once TMV has been assembled at the surface, Fig. 6b shows that electroless plating allows a thin (15 nm), confluent metal coating to be assembled onto this template (via the cysteine thiols). The images in Fig. 6c and d show the “carpet” of metal-coated virus templates that provides the large surface areas responsible for the observed 3 to 10 fold enhancements in anode capacity over commercially-available lithium ion based anodes.84,88,89

The above example illustrates multiple capabilities that are enabled by biotechnology’s fabrication toolbox. Recombinant technology allows protein building blocks to be site-selectively modified to impart specific chemical properties (e.g., the added cysteine residues provide sites for metal binding). A judicious combination of design and discovery allows the nano-environment of these “active sites” to be controlled to modulate activity (e.g., to attenuate surface binding). And the molecular-recognition-based self-assembly of the protein building blocks allows hierarchical structures to be generated. Importantly, hierarchical assembly is not limited to viruses90 but can be extended to other protein-based materials such as filaments (e.g., microtubules)91 and matrices.92

Tyrosinase-based enzymatic assembly

Biology routinely uses enzymes for the biosynthesis of soft matter (e.g., for proteins and polysaccharides) and there is considerable appeal to extending the use of enzymes for ex vivo soft matter fabrication.93-95 There are challenges that have limited these applications however. One challenge is that the enzymes employed for polymer biosynthesis require activated substrates and/or regenerable cofactors (e.g., ATP) which are inconvenient and/or costly for ex vivo synthesis. While the well-known hydrolytic enzymes that act on biopolymers (e.g., cellulases and proteases) do not require cofactors, they generally degrade biopolymers rather than build structure. Thus, despite the appeal, few enzymes have been employed to build macro-molecular structure.93,96-102 Our work has focused on two such enzymes.

Tyrosinases and related phenol oxidases are ubiquitous copper-containing enzymes that catalyze the oxidation of phenols into o-quinones using O2 as the co-substrate. o-Quinones are reactive electrophiles that can diffuse from the enzyme’s active site and undergo uncatalyzed reactions with various nucleophiles.103 Initial studies demonstrated that tyrosinase-generated o-quinones could react with chitosan’s nucleophilic amines104,105 and this enzymatic approach could be used to graft phenolic moieties to the chitosan backbone or to crosslink chitosan chains.106,107 Importantly, phenols are ubiquitous natural products and thus tyrosinase provides a means to access a diverse class of compounds to create chitosan derivatives with a range of functional properties (e.g., associative thickeners).108,109

Interestingly, phenolics are probably the most abundant redox-active compounds in nature110-112 and phenol-based biological materials (melanins111,112 and lignins113) have attracted attention for their “electronic” and opt-electronic properties.114-118 Previous studies have shown that the grafting of catecholic moieties to chitosan yields redox-active films that can be readily switched between oxidized (quinone; Q) and reduced (catechol; QH2) states as illustrated in Fig. 7a.119 Functionally, o-quinones are reactive electrophiles that can diffuse from the enzyme’s active site and undergo uncatalyzed reactions with various nucleophiles. Initial studies demonstrated that tyrosinase-generated o-quinones could react with chitosan’s nucleophilic amines104,105 and this enzymatic approach could be used to graft phenolic moieties to the chitosan backbone or to crosslink chitosan chains. Importantly, phenols are ubiquitous natural products and thus tyrosinase provides a means to access a diverse class of compounds to create chitosan derivatives with a range of functional properties (e.g., associative thickeners).108,109

Figure 7 Tyrosinase-catalyzed grafting of catecholic natural products confers redox properties. (a) Schematic illustrating the redox-capacitor capabilities of the catechol-modified film; the film can accept, store and donate the electrons. (b) A dual functional film uses glucose dehydrogenase (GDH) to harvest electrons from glucose and transfer them to the film by an NADPH redox-cycling mechanism.
that described in Fig. 3a).

The electrons to NADPH, while the NADPH undergoes the redox-reaction illustrated in Fig. 7b, that serves to “charge” the film by reducing the grafted chlorogenic acid moieties to their QH₂ state.¹²⁷

The above example illustrates that catecholic natural products confer redox activities to films. Importantly, these redox activities can be accessed by both electrochemical mediators and biological mediators (e.g., NADPH) and thus catechol-modified materials may offer a unique opportunity to bridge communication across a biology–electronics interface for applications in bioelectronics (e.g., for sensing and prosthetics).¹²⁸–¹³¹ In addition to redox activities, catecholic materials confer addition functional properties (e.g., adhesive)¹³²,¹³³ that are generating considerable recent interest.¹³⁴,¹³⁵

Importantly, tyrosinase is a versatile enzyme since it catalyzes reactions with both low molecular weight phenolics and also the phenolic moieties of macromolecules. In nature, tyrosinase oxidation of the dihydroxyphenylalanine residues of the mussel’s adhesive protein initiates crosslinking (i.e., setting) of this mussel glue.¹³⁶ Several groups have extended this observation by employing tyrosinase to oxidize tyrosine residues and initiate macromolecular grafting and cross-linking.¹³⁷–¹⁴⁰ While the tyrosine residues of open chain proteins (e.g., the adhesive protein and gelatin) are accessible for tyrosinase-mediated catalysis, the residues of globular proteins are less accessible (or inaccessible). While this may appear as a limitation, it also provides an opportunity. Specifically, if the gene coding for such a globular protein is engineered with a short tyrosine-rich fusion tag, then tyrosinase-mediated grafting may serve to conjugate and orient the protein through the fusion tag.¹⁴¹,¹⁴²

To illustrate this capability, Fig. 8a shows that the Streptococcal IgG-binding protein, protein G, was engineered to have a penta-tyrosine fusion tag that enabled its enzymatic assembly to chitosan; in this case to chitosan fibers. As illustrated in Fig. 8a, protein G binds to the constant (Fc) region of IgG antibodies and can orient these molecules for antigen recognition via their Fab binding sites. Thus this sequence of chitosan fiber formation, protein G grafting to the fiber (enzymatic assembly) and antibody binding to the protein G (self-assembly) provides a reagentless means to generate antibody-presenting fibers. As indicated in Fig. 8b, fibers can be combined to provide a simple platform (e.g., smart fabric) for multiplexed antigen detection.¹⁴³

**Transglutaminase-based enzymatic assembly**

A second enzyme used to build macromolecular structure is transglutaminase¹⁴⁶–¹⁴⁸ which crosslinks proteins by catalyzing the transamidation of glutamine and lysine residues to form N ε-(γ-gutamyl)lysine crosslinks.

The most familiar transglutaminase is the blood coagulation factor XIIIa that is responsible for fibrin crosslinking during clotting. A simpler microbial transglutaminase (mTG) has attracted considerable recent interest because it; does not require Ca²⁺, has few restrictions on the substrate’s sequence, can catalyze reactions with a broad range of proteins, and can accept alternative primary amines in place of the lysine residues. Thus, mTG is being studied to; generate crosslinked hydrogels,¹⁵⁵–¹⁵⁷ create dimeric proteins,¹⁶⁰,¹⁶¹ conjugate macromolecules,¹⁶²,¹⁶³ modify proteins site-selectively,¹⁶⁴,¹⁶⁵ and immobilize proteins.¹⁶⁶ As mentioned for tyrosinase, mTG reacts with amino acid residues that are accessible and thus

![Fig. 8](image-url) Tyrosinase-catalyzed grafting of the IgG-binding protein (protein G) to fabricate antibody-presenting fibers. (a) Schematic illustrating that a tyrosine-rich fusion tag allows for the tyrosinase-mediated grafting of protein G to chitosan fibers. (b) Antibody-presenting fibers can be multiplexed into smart fabrics.
most studies involve either open chain proteins (e.g., gelatin) or globular proteins that have been genetically engineered with short fusion tails to provide accessible glutamine and lysine residues. 

Fig. 9a illustrates the capability of engineering multiple biological functionalities into soft matter.\(^{168}\) In the first step, we enlisted the pH-responsive self-assembling small molecule hydrogelator 9-fluorenylmethoxycarbonyl-phenylalanine (Fmoc-Phe)\(^{71,169,170}\) to co-deposit gelatin along with \(E.\ coli\) reporter cells (the deposition solution was \(37^\circ C\) to retain gelatin in its soluble state). After cooling, gelatin forms its thermally-responsive physical gel and subsequent incubation of this matrix under slightly basic conditions (pH \(\simeq 7.4\)) allows Fmoc-Phe to leach from the matrix. In the second step, we added mTG along with two enzymes (designated Pfs and LuxS) each engineered with lysine or glutamine fusion tags. mTG performs two functions; it covalently crosslinks the gelatin network and it conjugates the enzymes to the network. Importantly, the mTG reaction is sufficiently mild to ensure the entrapped cells remain viable\(^{155}\) and the conjugated enzymes retain activity. 

To demonstrate both molecular (i.e., enzymatic) and cellular functions, we added the substrate S-adenosyl-homocysteine (SAH) which is sequentially converted to S-ribosyl-homocysteine (SRH) and 4,5-dihydroxy-2,3-pentanedione (DPD) by Pfs and LuxS, respectively. As illustrated in Fig. 9b, DPD undergoes a series of rearrangements to yield a family of compounds that are referred to as autoinducer-2 (AI-2) which is a bacterial quorum sensing signaling molecule. The entrapped reporter cells recognize AI-2 and respond by expressing the fluorescent protein DsRed. The results in Fig. 9c show that both enzymes are required for the entrapped cells to “report” the synthesis of AI-2.\(^{168}\)

The results in Fig. 8 and 9 illustrate that enzymes allow macromolecules to be assembled through the controlled introduction of covalent bonds and thus enzymatic-assembly provides an important complement to self-assembly. In particular, the mTG-crosslinking of gelatin creates a chemical gel that no longer dissolves at higher temperatures while the conjugates generated by tyrosinase and mTG are stabilized by their covalent linkages. We anticipate that the use of enzymes for materials synthesis will expand as more enzymes are explored for fabrication and as enzymatic fabrication steps are coupled.\(^{171}\) Other enzymes currently under investigation include laccases,\(^{172,173}\) peroxidases,\(^{174,175}\) and sortase.\(^{176}\)

**Engineering advanced function**

The above examples indicate that biology uses modules to confer functional properties. In some cases “native” modules have been found to be generically useful and have been incorporated into an ever-expanding biotechnology toolbox which also contains modules that have been purposefully designed/discovered (e.g., via synthetic biology).\(^{177,178}\) Examples of “assembly-modules” include; histidine tags that are routinely used to facilitate protein purification, cysteine residues that permit disulfide bond formation and metal-binding, the previously-discussed fusion tags that enable enzymatic conjugation, and amino acid sequences that promote self-assembly (e.g., leucine zippers\(^{179}\) or elastin-like polypeptides\(^{180,181}\)). While these examples illustrate a diversity of modules are available for assembly, biological modules can perform a wider array of functions.\(^{182}\) For instance, Fig. 10 illustrates the creation of a fusion protein that combines, in a single polypeptide chain, the Pfs and LuxS enzymes of Fig. 9, and the IgG-binding protein (protein G) and fusion tag of Fig. 8. The incorporation of an appropriate IgG antibody to this “nano-factory” leads to a protein assembly that can “target” the biosynthetic capabilities

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**Fig. 9** Fabrication of multi-functional matrix using microbial transglutaminase (mTG). (a) The stimuli-responsive film-forming hydrogelator (Fmoc-Phe) allows for the co-deposition of gelatin and reporter \(E.\ coli\), while mTG crosslinks (stabilizes) the gelatin matrix and conjugates two enzymes (Pfs and LuxS). (b) The matrix’s molecular (i.e., enzymatic) function is to synthesize the small bacterial signaling molecule (AI-2). (c) The matrix’s cellular function is to “report” the presence of the AI-2 signaling molecule by reporter cells that express the DsRed fluorescent protein in response to AI-2.
of the Pfs and LuxS enzymes to specific sites such as a bacterial cell,\textsuperscript{185} an epithelial cell surface\textsuperscript{186} or an address in a microfluidic device to elicit responses from nearby cells.\textsuperscript{187}

In general, Fig. 10 suggests the broader potential of biofabrication to enlist the emerging tools of synthetic biology (protein engineering, genome engineering and directed evolution): to enable the controlled incorporation of individual modules into hierarchical structures; to co-localize modules (e.g., enzymes in a biosynthetic pathway);\textsuperscript{187,188} or to permit targeting of autonomous units (e.g., functional nanoparticles). Additional functionalities could also be conferred by incorporating molecular modules for motors, switches and signaling motifs.\textsuperscript{189} And synthetic biology provides the opportunity to purposefully “design” entirely new modules that do not currently exist in nature. Thus, once the biotechnology toolbox can be fully accessed, then the opportunities for creating functional soft-matter will become nearly limitless!

Conclusions and outlook

Advances in modern biology transformed the life and medical sciences and are poised to make major contributions to materials science. In particular, biology has solved many of the challenges associated with mesoscale fabrication while the tools of biotechnology enable these solutions to be accessed, studied, manipulated and incorporated into soft matter. For instance, recombinant technology provides access to biology’s templated nucleic acid and protein biosynthetic processes that enable the generation of structure and function at the nanoscale. Self-assembly and enzymatic-assembly enable these nano-scale components to be “connected” over a hierarchy of length scales. Importantly, biology’s use of molecular recognition simplifies fabrication and reduces wastes by limiting the need for protection/deprotection steps that are common to chemical synthesis or the resists that are common to photolithographic patterning. Thus, while biofabrication approaches may be information-intensive, the ultimate protocols that emerge are simple, rapid and safe, and therefore should be readily transferrable into manufacturing. Also important is that many of the stimuli-responsive materials provided by biology (e.g., gelatin, alginate and chitosan) have a long history of use (e.g., in foods) and are often viewed as inherently safe platform materials for medical and pharmaceutical applications.\textsuperscript{192,193}

In summary, we anticipate the emergence of biology as an important enabler in materials science by complementing existing physical and chemical fabrication technologies, and by providing entirely new opportunities.

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