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Biofabricating Functional Soft Matter Using Protein Engineering to Enable Enzymatic Assembly

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Abstract

Biology often provides the inspiration for functional soft matter, but biology can do more: it can provide the raw materials and mechanisms for hierarchical assembly. Biology uses polymers to perform various functions, and biologically derived polymers can serve as sustainable, self-assembling, and high-performance materials platforms for life-science applications. Biology employs enzymes for site-specific reactions that are used to both disassemble and assemble biopolymers both to and from component parts. By exploiting protein engineering methodologies, proteins can be modified to make them more susceptible to biology's native enzymatic activities. They can be engineered with fusion tags that provide (short sequences of amino acids at the C- and/or N- termini) that provide the accessible residues for the assembling enzymes to recognize and react with. This "biobased" fabrication not only allows biology's nanoscale components (i.e., proteins) to be engineered, but also provides the means to organize these components into the hierarchical structures that are prevalent in life.

Graphical Abstract

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1. INTRODUCTION

Biology has already made significant contributions to materials science by providing the scientific underpinnings for the creation of functional materials (especially soft matter).¹ It is well-established that biology offers excellent lessons for soft matter fabrication, and various biomimetic approaches aim to understand and extend biology's ability to control structure at the nanoscale and assemble nanocomponents over a hierarchy of length scales. In addition to providing the inspiration for soft matter fabrication, biology also provides the materials and mechanisms that can be enlisted to provide an emerging paradigm for the construction of soft matter.^{2–5} There are several advantages for biobased fabrication. For example, biological materials such as polysaccharides and proteins are readily available from natural resources (i.e., abundant) and they are renewable and biodegradable (i.e., environmentally friendly). In addition, biological polymers often possess many unique properties (e.g., stimuli-responsive and self-assembling) that can be exploited for biologically based fabrication, biofabrication. Biological mechanisms (i.e., enzyme-catalyzed reactions) to macromolecular fabrication are often a “greener” alternative to conventional polymer synthesis approaches.⁶ From the standpoint of materials fabrication, enzymes allow the precise coupling of macromolecules (i.e., high selectivity)^{7–11} for the hierarchical assembly of biomacromolecules.^{12–17} The fact that enzymes catalyze reactions under physiological conditions can be exploited technologically^{18–26} and may offer opportunities in biotechnology (e.g., cells immobilization)^{27–32} and life science applications (e.g., drug delivery³³ and wound sealing).^{34–37}

In this review, we focus on the role of enzyme-catalyzed reactions for the biobased hierarchical assembly of soft matter and we especially focus on enzyme systems that are used in nature for organizing macromolecular structure. Many classes of enzymes, including oxidoreductases,^{38–42} transferases,^{43,44} hydrolases,^{45–47} and peptidases,^{16,48} have been explored as biocatalysts for the synthesis and modification of (bio)-polymers.^{9–11,49–52} However, only a handful of enzymes (as shown in Table 1) are known to cross-link and couple preformed biopolymers.^{10,12,43,53–55} Our work focused on two of the most studied enzymes: tyrosinases and transglutaminases.^{53,56,57} These enzymes have no cofactor requirements and thus are more readily adapted in practice. As will be discussed, these enzymes provide well-controlled routes to the hierarchical assembly of biological polymers to generate macromolecular architectures and can be enlisted to integrate with recombinant technology to confer biological functions.

Tyrosinases are copper-containing enzymes that use molecular oxygen to selectively oxidize a broad range of low molecular weight phenols as well as phenolic residues of proteins (e.g., tyrosine residues).^{58–60} As shown in Table 1, this enzyme catalyzes two reactions, the hydroxylation of phenols containing a single aromatic hydroxyl to form catechols, and the oxidation of catechols into *o*-quinones. The quinone is reactive and can diffuse from the enzyme's active site to undergo nonenzymatic nucleophilic reactions.^{11,60} In biology, tyrosinases (or phenol oxidases) mediate reactions that occur in a diverse range of processes including the browning of food,^{61–63} the synthesis of melanin,^{64–66} the sclerotization (hardening)⁶⁷ and sealing of wounds in the insect cuticle,^{68,69} and the curing of the mussel glue.^{70,71} As noted above, tyrosinases catalyze enzymatic conjugation without the need for

regenerable cofactors such as NAD(P)H. Unlike peroxidases, tyrosinases use O₂ rather than H₂O₂ as an electron acceptor and generate a reactive *o*-quinone intermediate rather than a free radical intermediate. A possible disadvantage of tyrosinase-mediated conjugation is that the *o*-quinone residues are reactive and can potentially undergo undesired side-reactions.

Transglutaminases catalyze the transamidation of glutamine (Gln) and lysine (Lys) residues to form *N*- ϵ -(γ -glutamyl)lysine cross-links⁷² as illustrated in Table 1. Probably the best-known tissue transglutaminase is Ca²⁺ dependent factor XIIIa that is responsible for cross-linking fibrin monomers during the late stages of blood coagulation.^{73,74} Tissue transglutaminases have various potential applications^{75,76} and fibrin-based medical sealants are commercially available,^{77,78} yet the high cost and the needs for Ca²⁺ ions and thrombin for activation limit their applications. Alternatively, a calcium independent microbial transglutaminase (mTG)^{43,79–85} is available that is less expensive and can function under a wide range of pH, salt content, and temperature (i.e., simpler to use). The mTG is expected to be safe because it has been developed for food and pharmaceutical applications.^{81,86–95} In addition, the mTG-catalyzed reaction is simple and leads to covalent bond formation with a broad range substrates.^{31,96–100} In comparison, sortase A (another commonly used enzyme for protein conjugation) catalyzes reactions that are prone to unwanted products due to the presence of different nucleophiles and hydrolysis of the recognition motif.¹⁰¹ Importantly, mTG catalyzed conjugation confers excellent selectivity because the nucleophilic attack by the lysine occurs at the enzyme's active site resulting in the direct transfer of the carbonyl from the thioester intermediate to the Gln residue.^{96,102–104}

2. IMPORTANCE OF PROTEIN ENGINEERING

Many proteins of interest (e.g., enzymes and antibodies) have compact globular structures and their amino acid residues may not be accessible for enzyme-mediated conjugation. To facilitate the enzyme-mediated conjugation of globular proteins, it is often desirable to “fuse” additional accessible amino acid residues to the protein of interest. This fusion can be achieved genetically by engineering the C- and/or N-termini of the protein to have a short unstructured amino acid sequence (i.e., a fusion tag). Figure 1 illustrates two examples of fusion tag proteins: N-terminus Tyr-tagged green fluorescent protein (GFP) that can be readily conjugated to stimuli-responsive aminopolysaccharide chitosan^{105–109} through tyrosinase-mediated reaction, and C-terminus Gln-tagged red fluorescent protein (RFP) that can be conjugated to lysine containing biopolymers through mTG-catalyzed reaction.^{29,96}

The use of fusion tags has been reported to offer three advantages. First, the conjugation efficiency of fusion tagged protein is typically more efficient than conjugation of the native protein, presumably because the tag provides readily accessible residues for conjugation.^{96,105,108,109} Second, protein engineering allows enzyme-mediated conjugation to be highly specific. For instance, when two structurally similar proteins (Tyr-GFP and Gln-RFP) were mixed at an equal molar ratio and incubated with mTG, the conjugation of Gln-RFP was found to be over 50 times higher than that of Tyr-tagged GFP. This result illustrates that mTG's chemoselectivity can control which protein is recognized and conjugated.⁹⁶ Third, the fusion tag may confer regioselectivity by controlling which part of the protein (the N- or C-terminus) is conjugated and this may control the orientation of the conjugated protein.¹⁰⁶

Taken together, the synergy of protein engineering and enzyme-mediated conjugation enables protein assembly with controlled selectivity and orientation.

3. TYROSINASE-CATALYZED CROSS-LINKING AND CONJUGATION

3.1. Tyrosinase-Catalyzed Conjugation of Small Molecules.

Tyrosinase enzymes are used by insects to harden or seal wounds to their cuticle.^{65,69,110,111} In this case, low molecular weight catecholics are oxidized to generate the reactive intermediate that is believed to cross-link the cuticle macromolecules (e.g., proteins and chitin).^{67,110} Figure 2a illustrates the tyrosinase-catalyzed grafting of low molecular weight catechols onto chitosan and the resulting catechol-modified chitosan films have found biomedical applications.^{112,113} Low molecular weight catechols such as caffeic acid and chlorogenic acid are abundant in nature.^{114–116} Chitosan, a β -1,4-linked linear copolymer of glucosamine and N-acetylglucosamine, is derived from partial deacetylation of chitin (the world's second most abundant polysaccharide). The primary amines ($-\text{NH}_2$) on the glucosamine residues of chitosan are nucleophilic and can readily react with *o*-quinones generated by tyrosinase-catalyzed oxidation of catechol,¹¹⁷ and the conjugation is believed to be the result of Schiff base and/or Michael type adduct linkages.^{118,119} In some cases, the *o*-quinones appear to be capable of cross-linking chitosan chains.^{120,121} The resulting catechol-chitosan films usually have altered optical, rheological, biological (e.g., antimicrobial and antioxidative), or even adhesive properties.^{118,122–124}

Recently, we found that chitosan hydrogel films grafted with various catecholics also have redox properties (i.e., they can accept, store, and donate electrons).^{125–130} For example, Figure 2b illustrates that a chlorogenic acid modified chitosan hydrogel film can (i) accept electrons from enzymatically generated NADPH to switch the film's redox-state from the oxidized quinone to the reduced catechol, and (ii) store these electrons in this reduced state.¹³⁰ Particularly, the film's ability to transfer electrons from a biological metabolite to an electrode suggests a potential applications in bioelectronics.^{131–133}

3.2. Tyrosinase-Catalyzed Protein Conjugation.

Tyrosinases can also oxidize accessible tyrosine (or dihydroxyphenylalanine) residues of proteins and this oxidation is integral to initiating the cross-linking reactions that serve to cure the mussel glue.^{70,71,134–136} Technologically, tyrosinases provide a mechanism for protein conjugation.^{60,137–142} Previous studies showed that the open chain protein gelatin can be conjugated to chitosan.^{143–146} Tyrosinases could also be used to create a protein–polysaccharide conjugate that has stimuli responsive “smart” properties. As illustrated in Figure 3a, tyrosinase-catalyzed conjugation reaction was performed by mixing engineered pentatyrosine fusion tagged GFP (Tyr-GFP) with chitosan under slightly acidic conditions (pH ~ 6) and adding tyrosinase to the solutions.¹⁰⁹ After reacting overnight at room temperature, the pH of the solution was raised to 9 to precipitate GFP–chitosan conjugate and a reducing reagent (NaBH_4) was added to irreversibly convert the Schiff base linkage. After centrifuging, the GFP–chitosan pellet was washed and redissolved in acid. The resulting GFP–chitosan conjugate offers pH responsive (i.e., “smart”) properties characteristic of chitosan.^{133,147} As shown by the photographs in Figure 3a, the GFP–

chitosan conjugate is soluble under acidic conditions (pH 5.5) but precipitates when the pH is raised to 7.2. Another useful property of GFP–chitosan conjugate is its ability to be assembled onto a patterned electrode surface in response to an applied voltage: this cathodic electrodeposition mechanism involves a pH-induced gelation through a neutralization mechanism.^{147–149}

Figure 3b demonstrates a fabrication strategy that integrates enzymatic assembly with directed assembly (i.e., electrodeposition) to confer antibody-based biorecognition function to a biochip electrode.¹⁰⁵ Specifically, a fusion of protein G with a tyrosine tag at the C-terminus was engineered and enzymatically conjugated onto a chitosan hydrogel film that had been previously electrodeposited on a gold electrode. Protein G recognizes and binds to the Fc-region of antibodies and, in this example, the anti-GFP antibody. The fabricated chips were then treated with various amounts of antigen (GFP). The fluorescence images of the chips were analyzed and the relative GFP binding efficiency was evaluated from the fluorescence. The plot (Figure 3b right) shows a semi-quantitative correlation between electrode's fluorescence and antigen concentrations in the incubation solution.

In sum, Figure 3 demonstrates that tyrosinase-mediated conjugation provides a simple and generic method to assemble target proteins onto a chitosan surface. This approach may be especially useful for conferring protein-based function to materials (e.g., for biosensor applications).¹⁰⁵

4. MTG-CATALYZED CROSS-LINKING AND CONJUGATION OF PROTEINS

4.1. mTG-Catalyzed Cross-Linking of Gelatin.

As previously mentioned, transglutaminase enzymes catalyze protein cross-linking reactions during blood coagulation and this enzyme has attracted attention for technological applications to cross-link and conjugate proteins. One protein of particular interest is gelatin which is a thermally responsive and gel-forming biopolymer that is widely used in the food and pharmaceutical industries.¹⁵⁰ At lower temperatures, gelatin self-assembles by forming reversible physical network junctions (noncovalent cross-links) due to the formation of collagen-like helices among different gelatin chains.¹⁵¹ Gelatin is also a useful structural biomaterial because it is an open-chain protein with accessible amino acid residues such as Lys and Gln. Figure 4a illustrates that when mTG is added to gelatin-containing solution, a cross-linked hydrogel network is formed. This mTG-catalyzed gelatin gel will not dissolve at elevated temperature as a result of the formation of covalent network junctions.^{152,153}

Interestingly, the mTG-catalyzed reactions have been considered for tissue adhesion because it allows bonding of gelatin to moist tissue and the adhesive strength is comparable to fibrin-based sealants.^{98,152,154} For example, the gel-forming process of the mTG-gelatin gels' capability to restrain fluid was tested and it was found that the adhesive and cohesive strength of the gel formed by mixing mTG and gelatin are high enough to restrain fluid at an average burst pressure of 320 mmHg, much higher than the physiological pressure (i.e., blood pressure).³⁵ The potential of the mTG-gelatin gel as a surgical sealant was also evaluated by performing in vivo studies in both small and large animal systems.³⁵ Figure 4b shows the results of a large animal study. First, an adult porcine's right femoral artery was

cut longitudinally to cause massive fountain bleeding; a clamp was used as a temporary hemostat and excess blood was removed. Then the mTG-gelatin mixture was applied to the wound site from a syringe (left photo in Figure 4b). After 4 min the clamp was gently removed and complete hemostasis was observed (right photo in Figure 4b), suggesting the mTG-gelatin has sealing capabilities. An obvious advantage of mTG-gelatin adhesive is its considerably lower cost compared with the fibrin-based sealants, which requires blood-derived components^{77,155} and tissue transglutaminase.^{76,156–159} In addition, the mTG-gelatin adhesive does not use blood proteins for hemostasis and thus avoids issues of the patient's coagulation state.^{160,161}

Since the mTG-catalyzed gelatin cross-linking occurs under mild conditions, it should allow viable components (i.e., cells) to be entrapped within the gelatin network.¹⁶² In a study shown in Figure 4c, *E. coli* cells were codeposited with gelatin on an ITO-coated glass slide.^{163–165} After deposition, the film was “set” at room temperature for 10 min and then incubated for 1 h in buffer (pH 7.4) containing mTG (1 U/mL) to cross-link the gelatin. The *E. coli*-containing mTG-gelatin film was then rinsed and incubated in LB medium at 37 °C, and the optical density was measured intermittently. The growth curve in Figure 4c shows a steady increase in optical density for the mTG-gelatin gels with entrapped bacteria, and the semilogarithmic plot suggests the entrapped population undergoes exponential growth with a doubling time of 1.5 h. The bright field image in Figure 4c indicates that cell growth is accompanied by the appearance of colonies (~20 μ m), presumably formed because of the restricted mobility of the cells within the mTG-gelatin gel network. The results in Figure 4c indicate that mTG-catalyzed gelatin cross-linking enables a viable bacterial population to be entrapped and cultivated within the gel matrix. These initial demonstration studies with mTG-catalyzed gelatin (and collagen) hydrogels have been extended to potential applications in tissue engineering^{34,51,162,166} and microfabricated fluidic channels.¹⁶⁷ Another interesting feature of the mTG-cross-linked gelatin gels are that simple protease-treatment can be used to degrade these hydrogels and release entrapped biocomponents (e.g., cells).

4.2. mTG-Mediated Protein–Protein Conjugation.

Conjugating proteins onto biopolymeric hydrogels is often required to confer biofunctionality and there is still considerable effort to develop simple and mild protein conjugation methods.¹⁶⁸ mTG provides one such approach by catalyzing amide bond formation between lysine and glutamine residues on proteins (as indicated in Table 1).^{7,24,169} Figure 5a illustrates an mTG-catalyzed conjugation of a globular protein onto the open chain protein gelatin. Specifically, a gelatin film was first electrodeposited onto a gold chip (similar procedure as described in Figure 4c).¹⁶⁵ Then, the gelatin-coated chip was incubated overnight at room temperature in a buffer solution containing red fluorescent protein engineered with glutamine tag (Gln₅) at the N-terminus (Gln-RFP) in the presence or absence of mTG. As shown in the fluorescent images in Figure 5a, Gln-RFP was assembled onto the gelatin film in the presence of mTG. After incubating in warm water (37 °C) for 2 h, the mTG-treated film shows minimal fluorescence change, indicating that a thermally stable (i.e., cross-linked) network has been formed. Thus, mTG provides a simple means to

both conjugate globular proteins to gelatin (to confer protein-based function) and covalently cross-link the gelatin chains (to confer thermal stability to the matrix).

mTG-catalyzed protein conjugation is not limited to gelatin, as recent studies have shown that proteins can be hierarchically assembled onto native spider silk fibers.⁹⁶ The schematic in Figure 5b shows that Protein G with Gln tag was conjugated to spider silk fiber via mTG-catalyzed reaction. As noted, Protein G binds antibodies and this conjugation approach allows the creation of an antibody-presenting silk fiber for antigen capture. In the demonstration study of Figure 5b, LacZ (β -galactosidase) was the antigen and binding of this enzyme could be observed by the hydrolysis of the colorless ONPG (α -nitrophenyl- β -galactoside) substrate into galactose (GAL) and the yellow-colored ONP (α -nitrophenol) products. The capture of LacZ by anti-LacZ conjugated spider silk and retention of its activity was observed by the colorimetric absorption at A₄₂₀ due to the generation of ONP. The right plot in Figure 5b shows that silk treated with LacZ and anti-LacZ showed higher hydrolytic activity of ONPG when compared to a control (a whole mouse IgG complexed that is not specific for LacZ) to Protein G-conjugated silk and treated with LacZ. This result demonstrates that mTG provides a versatile means to conjugate proteins to confer biofunctionality to materials.

5. FABRICATION OF COMPLEX SOFT MATTER

Hierarchically organized structures in biology (e.g., tissues and organs) are generally capable of performing diverse functions and there is growing interest in mimicking such systems in vitro. For instance, animal- or organ-on-a-chip devices are envisioned to provide an in vitro model for the discovery of drugs and characterization of their metabolism and toxicities.^{170–172} However, recapitulating the complex microenvironments of such biosystems (e.g., the gut)¹⁷³ remains a challenge, and a variety of biofabrication methods are being enlisted to achieve this vision.^{174–177} One important component of such complex biological systems is the molecular signaling that guides biological responses. For complex microbial systems (e.g., the microbiome), quorum sensing (QS)^{178–181} is a molecular communication modality that bacteria use to guide population-level behaviors.^{182,183} We are using protein engineering and enzymatic assembly methods to create hierarchical systems capable of engaging in QS. Specifically, we are engineering two enzymes, *S*-adenosylhomocysteine nucleosidase (Pfs) and *S*-ribosylhomocysteinase (LuxS), that are responsible for a short bacterial pathway for the synthesis of one such QS signaling molecule autoinducer 2 (AI-2). As shown in Figure 6, Pfs converts *S*-adenosylhomocysteine (SAH) to *S*-ribosylhomocysteine (SRH); LuxS converts SRH to homocysteine (HCY) and (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD), which is a precursor to AI-2. In our studies, we are engineering the Pfs and LuxS proteins to enable their enzymatic conjugation onto a diverse range of biomacromolecular matrices.

5.1. Assembly of QS Pathway Enzymes in a Multi-Subunit Protein Complex.

In our protein engineering approach, we use short linker tags (5–7 amino acids) to facilitate assembly.¹⁸⁴ As illustrated in Figure 7, one approach is to enzymatically couple individual enzymes into a multi-subunit protein assembly. In this example, the first subunit His-Pfs-Gln

was selectively bound to a solid support (Co^{2+} resin bead) via its N-terminal His tag. Then, the second subunit engineered with Lys tags on both termini (Lys-LuxS-Lys) was covalently grafted onto the Gln tag of the first subunit. Eventually, a subunit comprising a Gln tag on the C-terminus (e.g., Gln-LuxS) was used as the end unit of the assembly. In all cases, mTG was used to conjugate adjacent glutamine (Gln₅ tags) and lysine (Lys₇ tags) residues forming a trans-peptide bond.^{43,84,99,162,165} Using this approach, two and three subunit complexes comprising Pfs and LuxS enzymes were constructed.¹⁸⁴ In some cases, we replaced the Lys-LuxS-Lys with an *inactive* subunit LuxS obtained from periodic freeze–thawing. The enzymatic activities of these multi-subunit assemblies were quantified by adding the SAH substrate and measuring the formation of the HCY coproduct using real-time electrochemical measurements¹⁸⁵ (see Figure 6). The experimental results in Figure 7 shows that the highest metabolic fluxes were obtained for the Pfs-LuxS–LuxS trimer while the next highest reaction rate was observed for the Pfs-LuxS dimer. These results illustrate that the coupling of protein engineering and enzymatic conjugation enables the colocalization of the Pfs and LuxS biosynthetic enzymes into multi-subunit assemblies that may offer enhanced efficiencies through a channeling of pathway intermediates.

5.2. Co-Assembly of Individual QS Pathway Enzymes on Materials Platforms.

An alternative approach to colocalize the Pfs and LuxS pathway enzymes is to conjugate the individual enzymes to a materials platform. For instance, Figure 8a illustrates assembly onto the spider silk platform. In this example, equal molar amounts of Gln-tagged Pfs and Gln-tagged LuxS were mixed and conjugated to spider silk fibers via mTG-catalyzed conjugation. Again, Pfs and LuxS work sequentially to convert the SAH substrate to DPD which spontaneously interconverts into AI-2.^{186,187} In this example the pathway activity was measured by using a sulfhydryl assay (i.e., Ellman's Assay) for the HCY coproduct. The results in Figure 8a provide biochemical evidence for pathway activity while additional studies show these functionalized fibers could elicit appropriate biological responses (i.e., QS-induced bacterial chemotaxis).⁹⁶

In addition to using mTG to assemble the Pfs and LuxS enzymes onto a protein-based fiber (i.e., spider silk), it is also useful to assemble these QS pathway enzymes onto films and especially films of the polysaccharide chitosan. While the enzyme tyrosinase could be used to conjugate tyrosine tagged proteins to chitosan,¹⁰⁹ the tyrosinase-mediated oxidation of tyrosine residues to *o*-quinones can be slow compared to mTG-catalyzed reactions.^{188–190} Figure 8b illustrates a two enzyme assembly approach¹⁴⁶ that can accelerate conjugation of the QS enzymes onto chitosan films (and especially chitosan film coatings at an electrode surface).¹⁸⁸ In this example, a chitosan film was first electrodeposited onto gold chips^{3,147} and then the Lys-Tyr-Lys tripeptide was conjugated to the chitosan using tyrosinase.¹⁴⁶ In the second enzymatic step, mTG was used conjugate the Pfs and LuxS proteins that had been engineered with glutamine tags. Pathway activity was measured by incubating the SAH substrate (2 h at 37 °C) and measuring the HCY coproduct (by Ellman's assay). The results in Figure 8b demonstrate the use of these two orthogonal enzymes (tyrosinase and mTG) to create electrode coatings functionalized with the QS biosynthetic pathway.¹⁸⁸

5.3. Integrating Molecular and Cellular Functions to Synthesize and Recognize QS Signals.

As noted, gelatin is a common biologically derived material and mTG provides simple and biocompatible mechanisms capable of: conjugating proteins to confer molecular function (e.g., Figure 5a) and cross-linking the gelatin into a matrix with entrapped cells to confer cellular function (e.g., Figure 4c). These capabilities can be integrated as schematically illustrated in Figure 9. In this example, gelatin was co-electrodeposited¹⁶⁵ with *E. coli* reporter cells that respond to AI-2 by expressing red fluorescent protein. Initially, the deposited gelatin film is cross-linked through thermally reversible physical interactions (triple helices). This film was immediately treated with mTG plus fusion-tagged Pfs and LuxS proteins (either Lys-tagged or Gln-tagged) to allow the simultaneous cross-linking of gelatin to generate a stable covalently cross-linked matrix with entrapped reporter cells and conjugation of the Pfs and LuxS enzymes for biosynthesis of the QS signal. The resulting biofunctionalized films containing reporter cells were incubated in LB medium with the SAH substrate (18 h). The fluorescent images in Figure 9 indicate that the films conjugated with the Pfs and LuxS enzymes were capable of synthesizing the AI-2 signaling molecule and inducing fluorescent protein expression by the entrapped reporter cells.¹⁶⁵ This demonstration study illustrates the fabrication of a complex gelatin matrix with molecular and cellular functions that are capable of synthesizing molecular signals that induce a response from the biotic component of the matrix.

5.4. Biofabricate Protein-Based Molecular Communication Devices.

In recent years, there has been an emerging vision to extend the power of information technology to molecular-based communication modalities^{132,191–199} and to generate interconnected devices for an Internet of nanothings.^{200,201} This vision is stimulating efforts to create robust autonomous devices that can engage in molecular based communication.^{202,203} Potentially, stimuli-responsive self-assembling biopolymers can provide platform materials for such devices. As a proof-of-concept, we fabricated molecular communication devices from two stimuli-responsive biological polymers: the Ca²⁺-responsive polysaccharide alginate which can readily form hydrogel beads;^{204–212} and the thermally responsive protein gelatin.²⁹ Ca²⁺-alginate beads (including cell-containing beads) can be easily prepared by dropping an alginate solution into a stirring solution of CaCl₂. The limitations of Ca²⁺-alginate are that the stability of alginate beads can be limited, and subsequent covalent conjugation of proteins to biofunctionalize alginate can be problematic. As illustrated in the previous example gelatin may provide solutions for these limitations since mTG catalyzes gelatin-cross-linking that can confer stability and also protein–gelatin conjugation can confer protein-based molecular function.

To prepare a molecular communication device, Figure 10a illustrates that we engineered a multidomain fusion protein Gln-Pfs-LuxS to have two catalytic domains: Pfs-LuxS for synthesis of the AI-2 QS signaling molecule,²¹³ and a Gln₅ assembly tag to promote mTG-catalyzed conjugation to the gelatin matrix.^{213,214} Figure 10b illustrates that we prepared our bead devices from a warm prebead mixture containing gelatin, alginate, Gln-Pfs-LuxS, and mTG. Beads were formed by dropping this mixture into a CaCl₂ solution and then incubating for 2 h to allow mTG reactions to generate gelatin-cross-linked, Pfs-LuxS

conjugated beads. To demonstrate that these bead devices possessed molecular communication activities, they were transferred to a buffer solution containing the precursor SAH and the AI-2 reporter cells that had been engineered to constitutively express a red fluorescent protein (DsRed) and to conditionally express the enhanced green fluorescent protein (EGFP) in the presence of AI-2.²¹³ After incubation at 37 °C for 20 h, the surrounding medium was sampled and examined using a fluorescence microscope. The fluorescence images in Figure 10c show that when beads containing the Gln-Pfs-LuxS were present, the reporter cells in the surrounding medium expressed both red and green fluorescence. In contrast, when control beads that lacked Gln-Pfs-LuxS were used, these reporter cells showed red but not green fluorescence.

In sum, Figure 10 demonstrates that a soft matter based molecular communication device can be biofabricated using stimuli-responsive biological materials that are functionalized by enzymatically conjugating engineered proteins. This example illustrates the creation of bead-based devices that can add to the molecular communication of a surrounding bacterial population. It is also possible to use analogous devices to modify the molecular structure of the AI-2 QS signaling molecule and silence such cell–cell molecular communication (i.e., to “quorum quench”).²¹⁵

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Biology serves as an inspiration for materials science because it offers many lessons for creating structures and conferring properties that enable high performance functionalities. In particular, biology is unparalleled in its abilities to fabricate at the nanoscale with high precision and fidelity. For instance, proteins are synthesized with a precise sequence that programs folding into a stable three-dimensional structure that yields a binding site capable of molecular-level recognition. Further, biology is expert at assembling individual nanoscale components over a hierarchy of length scales to create systems capable of performing multiple complex functions. For instance, virus particles serve as nanoscale containers of genetic information that can evade immune defenses, gain access to cells, and assemble and disassemble as part of an infection cycle. Emerging research indicates that biology provides more than lessons for materials science; it can provide the materials and mechanisms for creating complex soft matter. Importantly, advances in biology (biotechnology and synthetic biology) are making these biological materials and mechanisms available to enable the de novo design and synthesis of materials. Also important is that biology performs its construction in aqueous solution under physiological conditions, and thus offers methods that are inherently biocompatible and safe. Finally, biology’s “products” are degradable and thus biological production provides a paradigm for sustainable manufacturing.

Here we summarize three important aspects of biological materials and mechanisms that can be extended to materials science. First are stimuli-responsive, self-assembling biological polymers such as chitosan, gelatin, and alginate that can undergo gelation (i.e., hierarchical assembly) in response to stimuli that are physiologically relevant. For instance, chitosan undergoes gelation when the pH is increased above ~6.2 and gelatin undergoes gelation when the temperature is lowered slightly below ambient conditions. Perhaps an underappreciated aspect of these stimuli (e.g., pH and temperature) responsive biopolymers

is that by user directed application of the stimulus, we can direct their assembly in geometrical configurations (length scales, patterns, shapes) that are not typically found in nature and at times controlled by device designers for use in specific applications. That is, we have shown how to assemble materials onto electronic devices²¹⁶ and how to electronically control their function.

Second, protein engineering allows access to biology's macromolecular synthesis machinery (i.e., transcription and translation) to allow non-native structures to be designed and created to perform specialized functions. For instance, short tyrosine-rich fusion tags function as "pro-tags": once activated by tyrosinase, they "instruct" the protein to undergo covalent grafting (e.g., to chitosan).¹⁰⁸ Also, protein fusions allow separate domains to be coupled into a multidomain protein to colocalize functions (e.g., to channel reaction intermediates). Third, enzymes can be used to catalyze covalent cross-linking and conjugation reactions that build macromolecular structure and confer biological functions. We focus on two such enzyme systems, tyrosinase and transglutaminase, which seem well-suited for in vitro applications because they both perform native functions outside the cell (i.e., without requiring regenerable cofactors) to create macromolecular networks to seal wounds. While these three aspects illustrate the potential for enlisting biology for materials science, we believe this is just a beginning and anticipate that the continued cross-fertilization between fields will enable capabilities that cannot yet be envisioned.

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Figure 1.

Fusion tags are short amino acid sequences that are genetically “fused” to a protein and these tags can facilitate enzymatic assembly. For instance, the pentatyrosine fusion tag (Tyr₅) on green fluorescent protein (GFP) facilitates tyrosinase-mediated conjugation and the pentaglutamine tag (Gln₅) on red fluorescent protein (RFP) facilitates transglutaminase-mediated conjugation.

(a) Tyrosinase-Catalyzed Catechol Conjugation on Chitosan **(b) Catechol-Chitosan Film Has Redox Activity** **Figure 2.**

Tyrosinase-initiated conjugation of low molecular weight catecholics. (a) Tyrosinase-catalyzed oxidation of phenols can generate *o*-quinones that readily graft to the primary amines on the chitosan backbone. (b) A redox-active catecholic-chitosan film has two functions: glucose dehydrogenase (GDH) harvests electrons from glucose, and NADPH shuttles them to the grafted catecholic moieties where the electrons are stored.

(a) Tyrosinase-mediated Protein-Polysaccharide Conjugation **(b)** **Figure 3.**

Tyrosinase-initiated conjugation of proteins. (a) Tyrosinase oxidation of accessible tyrosine residues can initiate protein conjugation to the polysaccharide (chitosan). The GFP–chitosan conjugate retains chitosan’s pH-responsive self-assembly properties that enable spatially selective electrodeposition. Adapted from ref 109 with permission from American Chemical Society. (b) Tyrosinase-mediated Protein G conjugation onto chitosan films allows antibody assembly and subsequent antigen capture. Adapted from ref 105 with permission from Wiley.

Figure 4. mTG-catalyzed cross-linking of gelatin to create a biocompatible matrix. (a) Schematic of gelatin cross-linking. (b) mTG-cross-linked gelatin gels as a surgical adhesive. Adapted from ref 35 with permission from Wiley. (c) Schematic illustrating an experimental procedure to demonstrate that bacteria can be entrapped and proliferate within mTG-cross-linked gelatin. Growth curve and semilogarithmic plot indicate exponential growth. Inset image shows entrapped bacterial colonies (~20 μ m) after incubation for 18 h in the gelatin gel. Adapted from ref 165 with permission from Wiley.

Figure 5. mTG-catalyzed protein–protein conjugation. (a) Gln tagged RFP conjugation onto electrodeposited gelatin. Adapted from ref 165 with permission from Wiley. (b) Protein G conjugated onto spider silk fibers to enable the creation of antibody-presenting fibers. Adapted from ref 96 with permission from Wiley.

Figure 6. Bacterial quorum sensing (QS) signaling molecule AI-2 is generated from a two-step pathway involving the enzymes, Pfs and LuxS.

Figure 7. Protein engineering coupled to enzymatic conjugation allows the creation of multi-subunit protein assemblies. Enzymatic activities of two and three subunit complexes. Adapted from ref 184 with permission from Elsevier.

Figure 8.

Co-localization of the QS biosynthetic enzymes on materials platforms. (a) Assembly of two pathway enzymes onto spider silk fiber to enable the biosynthesis of QS molecule. Adapted from ref 96 with permission from Wiley. (b) Two-step enzymatic assembly of QS biosynthetic pathway onto electrodeposited chitosan films. Adapted from ref 188 with permission from Springer Nature.

Figure 9.
Co-assembly of QS pathway enzymes and reporter cells on a material platform. Adapted from ref 165 with permission from Wiley.

Figure 10. Hydrogel-based molecular communication devices. (a) Engineered fusion protein Gln-Pfs-LuxS (fusions of the two biosynthetic enzymes and an assembly tag Gln). (b) Schematic illustrating that a hydrogel device with conjugated Gln-Pfs-LuxS can generate an AI-2 signal to “communicate” with reporter cells in the surrounding environment. (c) Fluorescence images of *E. coli* reporter cells from the surrounding environment: red fluorescence (DsRed) is constitutively expressed by both experimental and controls, while green fluorescence (EGFP) is only observed in the experimental samples. Adapted from ref 29 with permission from American Chemical Society.

Table 1.

Enzymes Frequently Used to Cross-Link Pre-Formed Biopolymers

Enzymes	Conjugating mechanisms
Tyrosinase
Laccase Peroxidase
Lysyl oxidase
Sortase
Transglutaminase