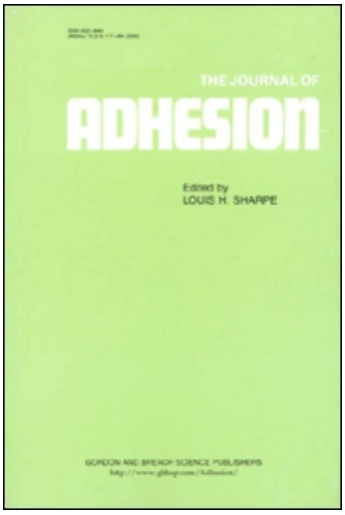

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Crosslinking Lessons From Biology: Enlisting Enzymes for Macromolecular Assembly

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Crosslinking Lessons From Biology: Enlisting Enzymes for Macromolecular Assembly

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Biology is well known for its use of linear polymers to perform sophisticated functions. Nucleic acids store and process genetic information, while proteins perform recognition, transport, and catalytic functions. Biology also employs polymers (especially proteins and polysaccharides) to perform mechanical functions and there are several examples in which biology covalently crosslinks polymers to confer elasticity and strength. In some cases, the crosslinking enzymes have attracted attention as a simple and safe means for macromolecular processing in vitro. *Here, we review recent research with two enzymes, tyrosinase and microbial transglutaminase, that are being examined for a variety of applications.*

Keywords: Chitosan; Crosslinking; Enzyme; Gelatin; Transglutaminase; Tyrosinase

INTRODUCTION

Professor Waite's study of marine adhesives pioneered research at the intersection of biochemistry and materials science. His work inspired others to understand and mimic the mechanisms that biology employs to perform mechanical functions. Here, we briefly review biology's covalently crosslinked polymers and we describe efforts to enlist such crosslinking enzymes *in vitro*. We especially focus on work with the enzymes tyrosinase and microbial transglutaminase to generate

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grafted and crosslinked polymers since these enzymes have been the focus of our group's research over many years.

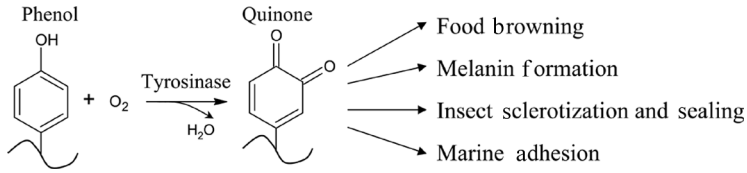
There are several potential motivations for employing enzymes for macromolecular processing. Enzymes are often viewed as "greener" alternatives to conventional catalysts and there has been considerable interest in enlisting enzymes for polymer synthesis and modification [1]. Enzymes catalyze reactions under physiological conditions and crosslinking enzymes may offer opportunities in biotechnology (*e.g.*, to immobilize cells) and medicine (*e.g.*, to seal wounds). And, enzymes offer selectivity that can be exploited for the precise coupling of macromolecules [2–4] or the hierarchical assembly of biomacromolecules to surfaces [5,6] (*e.g.*, to assemble proteins to device "addresses").

CROSSLINKED POLYMERS IN BIOLOGY

Polymer crosslinking is well known for the ability to impart elasticity and mechanical strength to polymeric networks and there are several examples of biological crosslinking. Bacterial cell walls are composed of peptidoglycan in which the polysaccharide network is strengthened by peptide crosslinks that are introduced by transpeptidase enzymes. The biological importance of this crosslinking is illustrated by the fact that the first commercial antibiotic, penicillin, functions by disrupting the bacterium's crosslinking reaction [7–9]. Similarly, the plant cell wall is composed of a polysaccharide (*i.e.*, cellulose) [10] that is crosslinked by other sugars (hemicellulose and pectin) [11] and, in some cases, by lignin [12]. While the crosslinked structure is not fully understood, a variety of enzymes are postulated to be involved, including peroxidases [13]. In animals, crosslinking more commonly involves proteins. For instance, the major structural polymer in mammals is the protein collagen, which can be crosslinked by the enzyme lysyl oxidase to enhance its mechanical strength [14]. A final example of biological crosslinking is the protein elastin which is reported to confer elasticity to connective tissue [15]. Of course, polymer crosslinking is not the sole means for obtaining mechanical strength as biology commonly employs minerals (*e.g.*, bones) to enhance mechanical properties.

TYROSINASE CATALYZED CROSSLINKING

Tyrosinases are copper-containing phenol oxidase enzymes that oxidize phenolic substrates using molecular oxygen as the oxidant. The products, *o*-quinones, are reactive and can diffuse from the enzyme's active site to undergo non-enzymatic reactions as suggested



Scheme 1 Tyrosinases catalyze phenol oxidation to form *o*-quinones that can undergo non-enzymatic reactions.

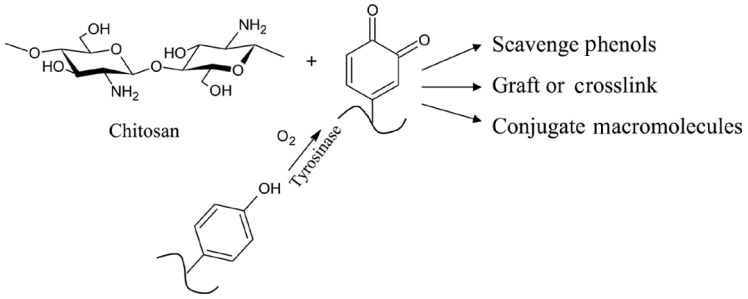
in Scheme 1 [16]. Typically, these reactions are responsible for the dark colors generated during such diverse processes as the enzymatic browning of food [17,18] and the synthesis of melanin pigments [19].

In insects, low molecular weight phenols (*e.g.*, tyrosine derivatives) are oxidized by tyrosinase to generate quinones that crosslink (and darken) the insect's cuticle in a process referred to as sclerotization or quinone tanning [20]. In addition, insects use tyrosinase to generate a crosslinked mesh that seals wounds [21,22] and they defend themselves by encapsulating foreign particles (*e.g.*, invading pathogenic bacteria) into melanin bodies [23–26].

Tyrosinases are also capable of reacting with protein substrates by oxidizing solvent-accessible phenolic residues such as tyrosine and dihydroxyphenylalanine (DOPA). The best-studied example is the tyrosinase-initiated crosslinking of the mussel's adhesive protein. The Waite group pioneered study of the "mussel glue" both to understand the biochemistry of the adhesive mechanism [27–29] and to apply this knowledge to technical adhesives that can cure under wet conditions [30]. Unfortunately, technical applications of the mussel glue have been limited due to the difficulties in extracting the mussel's adhesive proteins from natural sources and the challenges of expressing the adhesive protein through genetic engineering (the DOPA residue is not directly formed by the cell's protein translation machinery but must be generated through post-translational processing). However, these studies have stimulated biomimetic approaches in which phenolic residues are attached to macromolecular substrates and oxidation of these residues is used to initiate crosslinking or coupling [31–36].

Tyrosinase for Grafting and Crosslinking to Chitosan

Our group has been especially interested in coupling tyrosinase-generated quinones with the aminopolysaccharide chitosan as illustrated in Scheme 2. Chitosan is a β-1,4-linked linear copolymer of



Scheme 2 Coupling tyrosinase-generated quinones with chitosan.

glucosamine and N-acetylglucosamine that is obtained by the partial deacetylation of chitin (degree of acetylation is typically less than 20%). The primary amino groups of the glucosamine residues confer unique functionality to chitosan. In particular, these amino groups are nucleophilic and readily react with *o*-quinones generated by tyrosinase-catalyzed oxidation of phenols [37–39]. Initially, tyrosinase was envisioned as a means to activate troublesome phenols for their covalent attachment (*i.e.*, chemisorption) to chitosan. The oxidative reaction and strong adsorption drives phenol binding and, thus, could facilitate the high-affinity removal of polluting phenols from dilute solutions [40–45]. Compared with other adsorption methods (*e.g.*, activated carbon), the enzyme is chemically selective which offers interesting opportunities for chemical separations, to specifically remove an undesired phenol from a mixture of structurally-related non-phenols [46,47].

The observation that tyrosinase can initiate the covalent coupling of low molecular weight phenols onto chitosan suggested the potential for tyrosinase to generate grafted chitosans with structurally and functionally modified properties [48–50]. Importantly, plant biomass provides a particularly rich source of diverse phenols, many of which have been reported to have beneficial properties (*e.g.*, antimicrobial and antioxidant). Studies have shown that the enzymatic grafting of such natural phenols to the chitosan backbone can yield grafted polymers with altered rheological properties [51] or hybrid biological properties (*e.g.*, antimicrobial and antioxidative) [52]. For some phenols, the tyrosinase-generated *o*-quinones appear to be capable of forming multiple linkages (*e.g.*, they are bifunctional) and can then lead to the crosslinking of chitosan [53] or the coupling of another polymer (*e.g.*, a protein) to chitosan [54]. The potential of tyrosinase to generate low molecular weight *o*-quinone crosslinking agents has even been considered for adhesive applications [55,56].

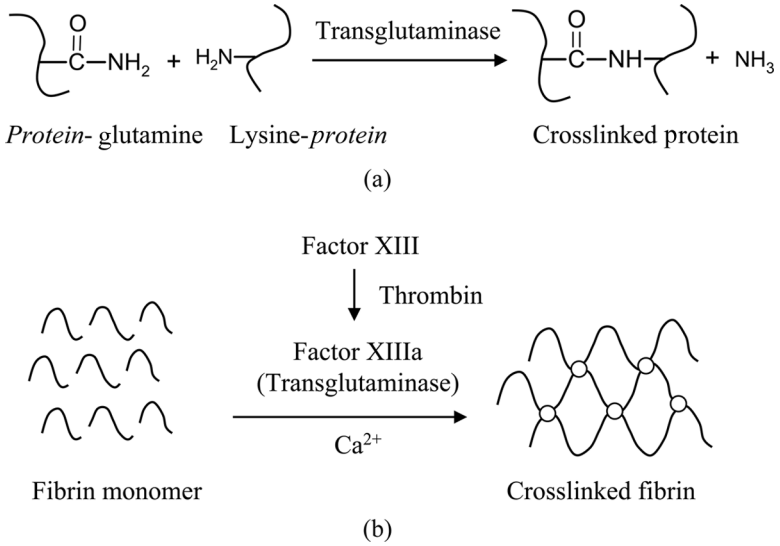
As mentioned, nature does not restrict the use of tyrosinase to low molecular weight phenols, as this enzyme can oxidize phenolic residues of macromolecules (*e.g.*, the tyrosine or DOPA residues of proteins) as in the case of the mussel glue. The capability of tyrosinase to activate macromolecular substrates has been considered as a means to graft peptides [57–59], proteins [60–65], and even synthetic polymers [66] to chitosan. One limitation of tyrosinase-mediated oxidation of macromolecules is that the phenolic residue must be accessible to the enzyme. In the case of the mussel glue, Waite's group showed that the adhesive protein has an open chain structure [67,68]. Interestingly, many proteins that perform structural functions also have open structures (*e.g.*, fibrous proteins) with many residues being solvent accessible. Not surprisingly, initial efforts to enlist tyrosinase to generate protein-chitosan graft copolymers have employed structural proteins (or derivatives of structural proteins). For instance, gelatin and silk fibroin were among the first proteins enzymatically grafted to chitosan.

Proteins that perform binding or catalytic functions (*e.g.*, antibodies and enzymes) often have highly compact, globular structures that lack surface-accessible tyrosine residues. Thus, the tyrosinase-mediated grafting of globular proteins to chitosan may require the addition of accessible phenolic substituents. This addition has been achieved genetically by engineering the gene for the protein so that when it is expressed in the host organism, the protein has a short, tyrosine-rich sequence fused to one of its ends. This sequence of fused amino acids is termed a fusion tag because it "tags" the protein with accessible residues for tyrosinase-mediated oxidation and subsequent conjugation to chitosan [69–73].

In summary, tyrosinase catalyzes the oxidation of low molecular weight phenols and accessible phenolic moieties of macromolecules and converts these phenols into reactive quinones. The broad substrate range of tyrosinase enables this enzyme to access the diverse array of phenols that are present in nature (*e.g.*, plant phenols) as well as the tyrosine residues of peptides and proteins. The quinones generated by tyrosinase readily react with the nucleophilic amines of chitosan to yield grafted polymers or crosslinked networks. Importantly, quinone-chitosan reactions are facilitated by the relatively low pKa (6–6.5) of chitosan's amines [74,75]. Also, protein-chitosan conjugates can be generated that possess chitosan's pH-responsive solubility [61].

TRANSGLUTAMINASE CATALYZED CROSSLINKING

Transglutaminases crosslink proteins by catalyzing the transamidation of glutamine and lysine residues to form N ε-(γ-glutamyl)lysine crosslinks as illustrated in Scheme 3a. Various mammalian transglutaminases are



Scheme 3 (a) Transglutaminases crosslink proteins by catalyzing the transamidation of glutamine and lysine residues. (b) Formation of crosslinked fibrin during the last stages of blood coagulation.

known and they appear to perform a variety of functions in tissue [76–78]. Probably the best studied transglutaminase is factor XIIIa that is responsible for fibrin crosslinking during the last stages of blood coagulation. As illustrated in Scheme 3b, the protease thrombin cleaves the inactive factor XIII into its active form factor XIIIa, which catalyzes the crosslinking of fibrin in the presence of Ca^{2+} [79]. Tissue transglutaminases have been studied for various potential applications [80–82] and the protein components of blood coagulation (*i.e.*, fibrin, thrombin, and factor XIII) are used clinically as fibrin sealants for surgical applications [83–86]. However, applications of tissue transglutaminases beyond medical applications have been limited by the cost of these blood- or animal-derived proteins and because of the needs for Ca^{2+} and enzymatic activation of the tissue transglutaminases (*e.g.*, the thrombin-catalyzed conversion of factor XIII to the active factor XIIIa).

Microbial Transglutaminase for Protein Grafting and Crosslinking

In the late 1980s, a screening effort in Japan yielded a microbial transglutaminase (mTG) that was smaller, had a broader substrate

range [87], and was calcium independent [88]. This enzyme is receiving considerable attention as a means to couple macromolecules and to generate crosslinked networks. Because mTG's coupling reaction takes place at the enzyme's active site, mTG can offer greater selectivity compared with tyrosinase, which generates a reactive intermediate that undergoes a non-enzymatic coupling reaction. Importantly, the mTG enzyme appears to be specific for glutamine although the requirement for lysine is more relaxed and mTG can accommodate a broad range of primary amines provided they have flexible alkyl linkers [89]. Like tyrosinase, however, the substrate residues must be accessible for mTG catalysis. Thus, while mTG is quite effective at crosslinking the open chain protein gelatin [90–94], it is less able (or unable) to react with globular proteins [95] unless their native structure is partially unfolded [96–98]. To facilitate mTG-catalysis with globular proteins, several investigators have genetically engineered the protein to have additional lysine or glutamine residues (*e.g.*, fusion tags) at the amino and/or carboxy termini of the protein [99–104].

Much of the initial studies with mTG focused on its ability to crosslink peptides and proteins for food applications [105–108]. However, the ability of mTG to generate crosslinked networks without the need for reactive reagents attracted broader attention as illustrated in Table 1 [109]. For instance, the mTG catalyzed crosslinking of gelatin is sufficiently mild that it can be used for the *in situ* entrapment of cells for biotechnology applications. Bacterial cells that were *in situ*-entrapped within an mTG-crosslinked gelatin gel were shown to be viable and capable of growing and responding to environmental cues (*e.g.*, inducers). Further, the entrapped cells could also be released by the proteolytic degradation of the crosslinked gelatin network using standard protease enzymes [110].

The mTG-catalyzed crosslinking reactions have also been extended to mammalian cells, suggesting the potential of this covalently crosslinked network as a scaffold for tissue engineering applications. In some cases, the mTG-catalyzed sol-gel reaction has been examined

TABLE 1 Potential Applications of Calcium-Independent Microbial Transglutaminase (mTG)

Food	Enhance texture and stability of protein-rich foods
Biotechnology	Generate matrix for cell immobilization
Medicine	Create scaffold for tissue engineering
	Cure soft tissue adhesive/sealant
Microfabrication	Fabricate soft surfaces
	Assemble proteins at surface

for *in situ* entrapment of cells [111,112]. In other cases, mTG crosslinking has been used to enhance the mechanical properties of gelatin (or collagen) networks to generate robust scaffolds for cell proliferation [113–115]. Importantly, gelatin and collagen have reasonably long track records for safe use in medical applications (*e.g.*, as surgical adhesives).

The mTG-catalyzed crosslinking of gelatin has also been suggested as a soft tissue adhesive [116–118]. The crosslinking reaction between gelatin and mTG is directly analogous to the fibrin crosslinking catalyzed by factor XIIIa that occurs during the last stages of the blood coagulation cascade. This gelatin-mTG biomimetic adhesive cures under moist conditions over the course of a few minutes and confers cohesive and adhesive strength appropriate to bond tissue. Potentially, the gelatin-mTG biomimetic adhesive could compete against fibrin sealants for medical (*e.g.*, surgical) applications. Compared with fibrin sealants, the gelatin and mTG components should be readily available (they are not derived from blood) and less expensive. Also, the gelatin-mTG biomimetic adhesive may be simpler to use since crosslinking occurs independent of thrombin and Ca^{2+}. Obviously, the biocomaptability of mTG would need to be tested to ensure its safety in *in vivo* applications.

In addition to generating crosslinked networks, mTG is also being explored as a means to graft substituents to proteins to tailor properties or confer hybrid properties. For instance, mTG has been utilized to site-selectively modify proteins with amine-functionalized PEGs [119] and sugars [120,121], and to conjugate other small molecules (*e.g.*, haptens) to proteins [122,123]. Further, mTG has been employed to generate heterodimeric proteins [99–102,124], to immobilize proteins [103,104], and to couple proteins to other biological polymers (*e.g.*, carbohydrates [120,125–127] and DNA [128]) that have been suitably modified to serve as a substrate for mTG.

The above examples illustrate that enzymes provide unique opportunities for the macromolecular construction of soft materials with the most obvious applications in food, biotechnology, and medicine. With the growing interest in coupling biology with electronics (*e.g.*, for biosensing and high throughput experimentation), there is an emerging interest in integrating soft materials and enzymes into device fabrication [129].

One set of studies has shown that the mTG-catalyzed crosslinking of gelatin can be used in molding operations to generate soft microchannels that can be used to cultivate cells in controlled microenvironments [130,131]. Another study has shown that mTG can be used for conjugating proteins to surface sites at electrode addresses [132].

In summary, mTG offers a means to crosslink or couple proteins through accessible glutamine and lysine residues. Since mTG reacts with a broad range of primary amines (and not just lysine residues), this enzyme also allows small or large molecules to be grafted to proteins with residue-specificity. Importantly, mTG does not require reactive reagents or activated substituents and, thus, mTG provides a simple and safe method for coupling and crosslinking. As a result, the initial applications for mTG are expected to be in food, biotechnology, and medicine. However, the simplicity and residue-specificity of mTG suggest this enzyme can be used for the precise construction of macromolecular assemblies for a broader range of applications.

OUTLOOK

Prof. Waite's work on mussel adhesion has demonstrated that biology offers many important lessons for materials scientists. Biology's crosslinking enzymes may enable us to apply two such lessons. The first lesson is sustainability. Enzymes enable renewable, bio-based raw materials to be accessed to generate functional materials. We are using various natural phenols, proteins, and polysaccharides to create functional polymers and crosslinked networks. In addition to the renewable raw materials, the enzyme-catalyzed processes are expected to be inherently safe and the products environmentally-friendly (*e.g.*, biodegradable). The second lesson is bottom-up precision assembly. A current challenge in nanotechnology is the hierarchical assembly of pre-formed nanoparticles into precise assemblies for either drug delivery or nanoelectronics. Nature is well known for its ability to employ non-covalent mechanisms (*e.g.*, hydrogen bonds and hydrophobic interactions), molecular recognition, and self-assembly to precisely construct macromolecular assemblies from their component parts. Enzymes are capable of selectively adding covalent bonds which could stabilize macromolecular assemblies and, thus, complement self-assembly methods based on reversible physical mechanisms. As we learn these lessons, we expect a growth in the list of examples in which biology's crosslinking enzymes are explored for technological applications.

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