Received: 27 April 2020 | Revised: 13 June 2020 | Accepted: 15 June 2020

DOI: 10.1002/bip.23390

REVIEW

Biopolymers WILEY

Enzyme-catalysed polymer cross-linking: Biocatalytic tools for chemical biology, materials science and beyond

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Funding information
Biotechnology and Biological Sciences Research Council, Grant/Award Numbers: BB/L01386X/1, BB/M012107/1, BB/M025624/1, BB/T001968/1; Engineering and Physical Sciences Research Council, Grant/Award Numbers: EP/L016494/1, EP/R020957/1; University of Bristol

Abstract

Intermolecular cross-linking is one of the most important techniques that can be used to fundamentally alter the material properties of a polymer. The introduction of covalent bonds between individual polymer chains creates 3D macromolecular assemblies with enhanced mechanical properties and greater chemical or thermal tolerances. In contrast to many chemical cross-linking reactions, which are the basis of thermoset plastics, enzyme catalysed processes offer a complimentary paradigm for the assembly of cross-linked polymer networks through their predictability and high levels of control. Additionally, enzyme catalysed reactions offer an inherently 'greener' and more biocompatible approach to covalent bond formation, which could include the use of aqueous solvents, ambient temperatures, and heavy metal-free reagents. Here, we review recent progress in the development of biocatalytic methods for polymer cross-linking, with a specific focus on the most promising candidate enzyme classes and their underlying catalytic mechanisms. We also provide exemplars of the use of enzyme catalysed cross-linking reactions in industrially relevant applications, noting the limitations of these approaches and outlining strategies to mitigate reported deficiencies.

KEYWORDS

biocatalysis, bioconjugation, covalent bond, cross-linking, polymeric materials, thermosetting polymers

1 | INTRODUCTION

Cross-linking reactions are a cornerstone of polymer science. They enable the targeted assembly of unique hierarchical structures whose physiochemical properties are distinct from those of the parent monomer or monomers. The formation of covalent bond linkages between polymer chains creates multidimensional polymer architectures with desirable properties at the molecular, nanolevel, microlevel and macrolevel. Examples of frequently observed desirable bulk characteristics include profound changes in T_g, melting temperature, solubility and elasticity, as well as the provision of notable mechanical strength and resistance to wear.[1,2] Consequently, the judicious use of intermolecular cross-linking reactions has provided access to a plethora of functionally diverse polyurethanes, vulcanised rubbers, epoxy resins and polyesters, which offer robust alternatives to their thermoplastic analogues.

The development of selective yet robust methods for polymer cross-linking has been a major focus of research effort for decades. Current favoured methods include the introduction of chemical cross-

This review is dedicated to the memory of Dr Robert Downs (1986-2020). A gifted scientist and exceptional colleague, whose warmth, thoughtfulness and good humour will never be forgotten.

linkers, the use of high-energy irradiation, photosensitisers in combination with visible or UV light to initiate radical based processes, or extremes of temperature and pressure to drive additional reactions.[3–5] In contrast, enzymatic cross-linking reactions have been less extensively employed in polymer science, despite being the cross-linking method of choice in biological systems. Enzymatic methods offer highly selective, atom efficient catalysis for intermolecular covalent bond formation, utilising nature's panoply of substrates, target functionality and biocatalysts, all under mild reaction conditions akin to those tolerable by the host organism.[6] In addition, the sourcing of biocatalysts from natural organisms makes them by definition inherently less reliant on toxic agents to achieve cross-linking efficiency, translating into processes with significantly reduced environmental impact. Importantly, modern biomanufacturing methods are driving down the cost of enzyme production, making them increasingly more economic in comparison to abiotic alternatives, especially if reduced processing and waste disposal costs (as well as the implied use of more biosourced and biodegradable components) are included in the life cycle assessment.[7]

The value of enzymes as cross-linking agents for polymers is founded on a sizeable body of work exploring biocatalytic covalent bond forming reactions in biology and biotechnology. Enzyme catalysed cross-linking can be categorised into two modalities: (a) direct covalent bond formation between partner molecules, as is common in cross-linking reactions catalysed by transferases or hydrolases[6] and (b) enzyme-mediated covalent bonding, where enzymes direct the interpolation of reactive species, which subsequently react spontaneously to generate a covalent bond, as is commonly seen in cross-linking reactions catalysed by oxidoreductases.[6] Importantly, either modality has the potential to provide biomimetic approaches to unlock new polymeric architectures, networks and materials.

In this review, we summarise the current state-of-the-art in biocatalytic cross-linking as applied to biological, synthetic and hybrid polymer systems, focusing on the most promising candidate enzyme classes and their mechanistic scope. We provide exemplar use cases which highlight the complementarity of enzyme-based approaches to established chemocentric methods for polymer cross-linking. We also identify instances where biocatalytic cross-linking has the potential to transform current approaches in polymer chemistry, while recognising potential drawbacks and proposing routes to their circumvention.

2 | TRANSGLUTAMINASE—THE POLYMER SCIENTIST'S FLEXIBLE FRIEND

Transglutaminases (EC 2.3.2.13) are a family of well-studied enzymes common to both eukaryotes, archaea, and bacteria. They have been the subject of considerable investigation over many decades, with studies focusing on both the delineation of their catalytic mechanisms and their specific functions in biological systems.[8] They also represent one of the few examples of a biocatalytic cross-linker currently industrially exploited at scale, for example, as a cross-linking ingredient in the culinary product Meat Glue, which is widely used to cross-link proteins in both processed meat products such as chicken nuggets, and in gourmet restaurants to create novel food combinations and textures.[9,10] In eukaryotic systems, transglutaminases are widely distributed in both the skin and brain,[11] where they catalyse calcium-dependent cross-linking resections

TABLE 1 Natural functions of cross-linking enzymes

Enzyme	Biological function	Ref.
Human transglutaminases	Transglutaminase 1 cross-links membrane and desmosomal proteins in cell envelope formation. Transglutaminase 4 coagulates semen and has an essential role in male fertility. Mammalian fibrin-stabilising factor XIII cross-links fibrin chains in blood coagulation, functions as a cell-adhesion protein and matrix cross-linker in tissue repair and cell death, and cross-links osteopontin in bone growth.	[6,8,13,14,83–87]
Microbial transglutaminase	Involved in the differentiation and spore surface formation of *S. hygroscopicus* and participates in cell wall formation in methanobacteria. Cross-links cell wall proteins in *C. albicans* and *S. cerevisiae* and cross-links spore coat proteins in *B. subtilis*.	[88–90]
Human tyrosinase	Oxidises L-tyrosine to dopaquinone, which undergoes subsequent reactions to produce eumelanin, or spontaneously cross-links with cysteine to produce pheomelanin in melanogenesis.	[67]
Mushroom/apple tyrosinase	Oxidises phenolic compounds to quinones, which spontaneously cross-link to form melanin pigments in enzymatic browning.	[68,91]
Insect tyrosinase	Oxidises L-tyrosine to dopaquinone which spontaneously cross-links with cysteine or glutathione to produce melanin in the insect immune response.	[92]
Sortase class A	Ligates secreted proteins containing a cell wall sorting signal to a polyglycine cell wall component; cell wall precursor lipid II.	[33]
Sortase class C	Polymerises pilin subunits in Gram-positive bacterial pili formation.	[93]
Lysyl oxidase (mammalian)	Cross-links collagen and elastin fibres in ECM remodelling.	[61]

Abbreviation: ECM, extracellular matrix.

including those involved in blood clot formation through fibrin cross-linking and the maintenance of tissue integrity (Table 1).[6,12–14]

Much of what is known about eukaryotic transglutaminases has been derived from studies of fibrin-stabilising factor XIII. This enzyme catalyses the introduction of intermolecular covalent bonds between glutamyl and lysyl side chains in protein and peptide substrates (Figure 1). The reaction proceeds via the formation of a covalent proteinyl-enzyme-thioester intermediate from a glutamyl motif on one chain, facilitated by a cysteine, aspartate and histidine triad.[15] The lysyl ε-amino group from another chain then initiates a nucleophilic attack on the thioester carbonyl, which resolves the enzyme bound intermediate and liberates a covalently cross-linked product from the transglutaminase active site.[6] Importantly, there are minimal restrictions on the precise location of addressable glutamine and lysine residues within substrate molecules, thus fibrin-stabilising factor XIII displays cross-linking activity with a myriad of non-cognate substrate pairs.[6] Recently, a cold adapted transglutaminase has been reported from the Atlantic cod, which demonstrates high catalytic efficiency at low temperatures (8°C-16°C). This has the potential for use in the processing of chilled foods, where the higher temperatures currently required for transglutaminase activity can lead to food spoilage.[16] The adoption of fibrin-stabilising factor XIII and its eukaryotic relatives as generic cross-linking agents has, however, been limited by both its calcium dependency and challenges associated with its large-scale manufacture in recombinant form, issues that have been addressed through the use of prokaryotic transglutaminases.

In 1989, Ando *et al* provided the first evidence that microbial transglutaminases, unlike their eukaryotic equivalents, are calcium independent enzymes.[17] This lack of cofactor dependency has since been shown to be common to all prokaryotic transglutaminases.[6] Although microbial transglutaminases share little sequence identity with their eukaryotic counterparts, a consequence of their distinctive single rather than four domain structure,[18] they do possess an analogous catalytic triad and general active site architecture (Figure 2).[19]

The use of microbial transglutaminase as a biocatalytic cross-linker was initially proposed by Hiroshi *et al*,[20] and optimised variants of this enzyme were subsequently patent-protected.[21] The crystal structure of the *Streptoverticillium mobaraense* transglutaminase was elucidated in 2002,[22] which has proved critical in further functional optimisation and structural stabilisation of microbial transglutaminases (Figure 2). This development has unlocked a raft of potential applications of transglutaminase as a polymer cross-linker for both natural and synthetic polymers, including its use in areas as diverse as food restructuring and biosensing (Table 2).[15,23–26] The suitability of *S. mobaraense* transglutaminase for large-scale recombinant production has seen it widely adopted in industrial cross-linking processes. Also enhancing its commercial potential are the enzyme's broad pH and temperature range tolerance[6] and its classification as non-toxic and non-immunogenic by the FDA, making it suitable for use in pharmaceutical and agritech applications.[18] It has, however, recently been implicated as potentially immunogenic to celiac patients.[27] One notable commercial application is the use of transglutaminase cross-linking during the manufacture of machine washable wool.[28] Following transglutaminase treatment and subsequent keratin-fibre cross-linking, wool exhibits a significantly greater resistance to repeated washing cycles with proteinase-based detergents and an increased tolerance to hydrogen peroxide bleaching.[29]

In parallel with advances in our fundamental understanding of transglutaminase (bio)chemistry, significant progress has also recently been made in broadening the diversity of this enzyme class. Through the use of large-scale environmental sampling and the application of protein engineering, it has been possible to isolate new microbial transglutaminases with enhanced kinetic parameters and improved chemical and thermal tolerances, for example, the *Streptococcus suis* transglutaminase; though a truly thermophilic microbial transglutaminase has yet to be formally reported.[30,31] In addition, a collection of recently identified bacteria have been shown to possess the capacity to secrete transglutaminase at high yields.[18,19] It is hoped that the use of these strains will enable a reduction in the cost of the manufacture of high purity transglutaminases due to decreased requirement for downstream processing. Recently, Duarte *et al* have published two reviews examining the origins and applications of transglutaminases where they discuss in depth their biological functions, as well as the optimal conditions for these enzymes from various organisms involved in many of the applications listed in Table 2.[32]

3 | SORTASE—MORE THAN JUST ANOTHER BRICK IN THE (CELL) WALL

Sortases (EC 3.4.22.70) are a group of cysteine transpeptidases primarily found in Gram-positive bacteria. They catalyse the formation of an amide bond between a cell wall sorting signal located on the C-terminus of a polypeptide substrate, and an exposed poly(glycine) group present on a secondary substrate.[33] The best studied sortases are those of the sortase A (SrtA) class, the so called 'housekeeping' sortases, which are common to Gram-positive bacteria and recognise a distinctive Leu-Pro-X-Thr-Gly (LPXTG) sorting signal.[33,34]

SrtA transpeptidase activity involves cleavage of the peptide bond between the threonine and glycine residues within the substrate sorting signal, forming a thioester intermediate within the enzyme active site. This intermediate undergoes subsequent nucleophilic attack by the N-terminal glycine of the secondary substrate, resulting in the formation of an amide bond between the threonine of substrate 1 and the glycine of substrate 2 (Figure 3).[35] Despite SrtA enzymes being membrane-bound proteins, it has proven routinely possible to express their catalytic domains as isolated soluble recombinant polypeptides at high yield.[36,37] Although natural SrtA enzymes are known to require bound calcium ions for structural integrity, mutagenesis studies have identified engineered SrtA variants lacking this requirement. These proteins are stable, and do not exhibit a loss in enzyme activity following incubation at room temperature for >24 hours.[38,39]

The ability of sortases to proficiently fuse proteins or peptides to poly(glycine) containing substrates has encouraged researchers to explore the use of these enzymes across a range of application areas.[40–42] It should be noted that the enzyme in most of these

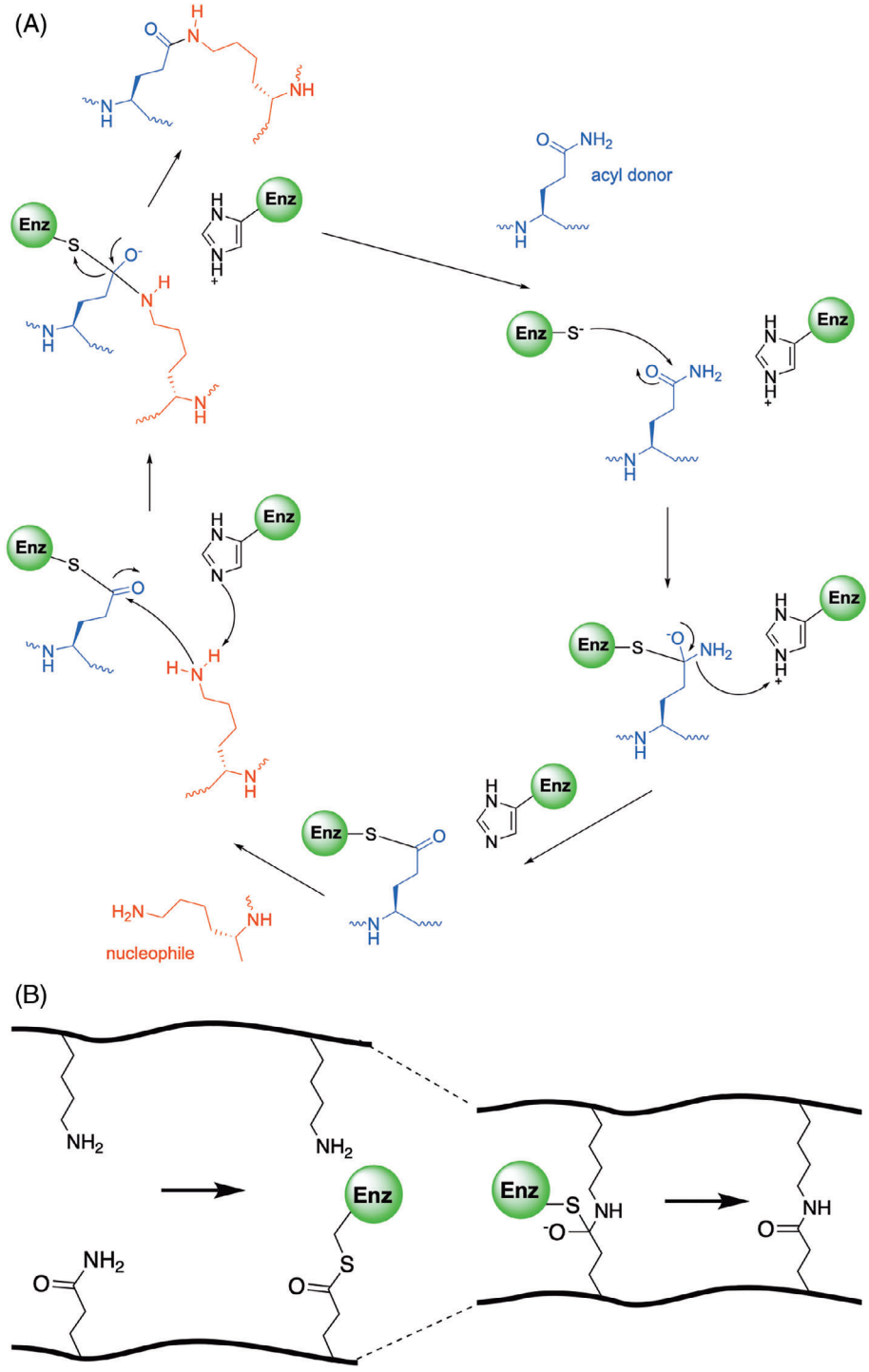

FIGURE 1 A, Catalytic mechanism of transglutaminase.[189] The enzyme catalyses intermolecular covalent bond formation between substrate glutamyl and lysyl side chains. The reaction proceeds via the formation of a covalent proteinyl-enzyme-thioester intermediate from a glutamyl motif on one chain, facilitated by a cysteine, aspartate and histidine catalytic triad. The lysyl ε-amino group from another chain then initiates a nucleophilic attack on the thioester carbonyl, resolving the enzyme bound intermediate and liberating the cross-linked product from the enzyme active site. (B) Schematic representation of transglutaminase catalysed polymer cross-linking

applications is linking linear segments to create a longer chain rather than catalysing cross-linking between chains through non-terminal locations. However, sortases have shown sufficient promiscuity of substrate acceptance both within and between sortase classes, to enable them to adopt the role of biocatalytic cross-linkers as well.[35] Directed evolution of sortase A has also been shown successful in both broadening and altering substrate promiscuity through enabling recognition of alternative sorting signals.[43,44]

Sortase enzymes have been shown to accept non-peptidic components enabling the modification of non-proteinogenic polymers and hydrogels, and this has been investigated in some detail.[45,46] SrtA catalysed polymer cross-linking has been used for the generation of hydrogels for human tissue culture, as first demonstrated by Arkenberg and Lin in 2017.[38] In this study, a non-calcium-dependent mutant of *S. aureus* SrtA with increased enzyme activity was used to connect a PEG polymer construct with a pendant LPRTG sorting signal to polyglycine. More recently, these results have been reproduced in a hyaluronic acid-based polymer system.[39] Notably, and with respect to the wider potential applications of this technology, the purity of the recombinant SrtA employed in these studies enabled the formation of cross-linked

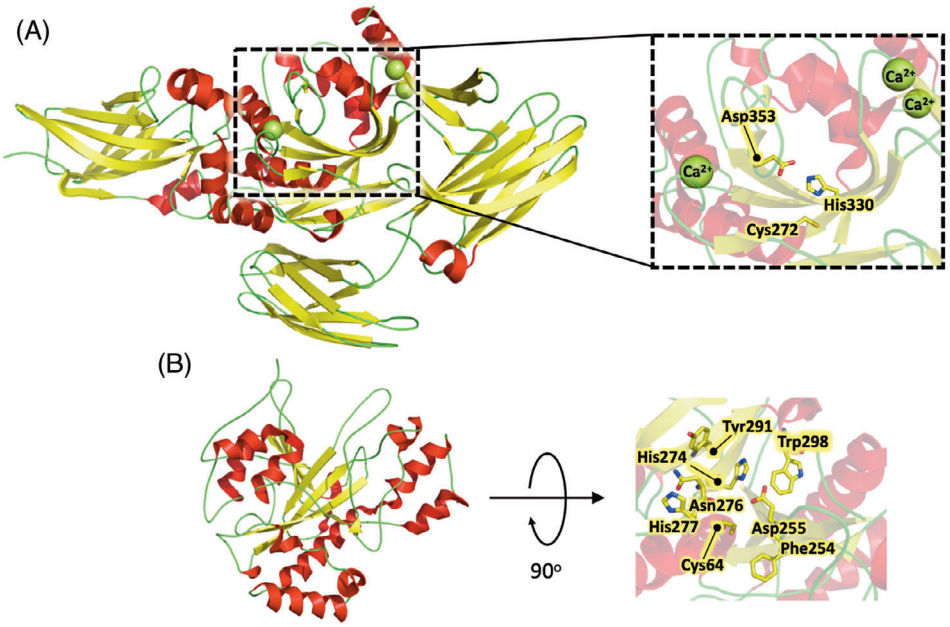

FIGURE 2 Crystal structures of eukaryotic and prokaryotic transglutaminases. A, Overall fold of the active form of human transglutaminase 3 (PDB, 1NUD). Inset, enzyme active site highlighting the residues which constitute the catalytic triad. The location of the three bound calcium ions is also highlighted.[190] B, Overall fold of the calcium independent *S. mobaraense* transglutaminase (PDB, 1IU4). Also shown is the composition of the enzyme active site, highlighting resides proposed to contribute to substrate binding and/or catalysis[22]

hydrogels possessing endotoxin concentrations well within FDA approved limits. Given that the microbial SrtA LPXTG sorting signal is not recognised or processed by mammalian cells, and SrtA has no known native mammalian protein substrates, SrtA cross-linked hydrogels appear ideally suited for use in human cell culture. Importantly, the functional properties of SrtA hydrogel scaffolds such as stiffness have also been shown to be comparable to those produced by Matrigel, or chemically cross-linked PEG-based hydrogels.[38,39]

One additional intriguing observation is that SrtA may also be employed as an agent of hydrogel dissolution (Figure 4).[47] Valdez *et al.* demonstrated the utility of a synthetic PEG-norbornene extracellular matrix (ECM) hydrogel cross-linked by peptides containing an LPXTG motif, within which the threonine and glycine bond could be cleaved by SrtA. Epithelial and stromal cells cultured on this hydrogel could be readily recovered in high yield from this scaffold via sortase-mediated dissolution. This contrasts with traditionally employed approaches that rely on the use of proteases to dissolve the support matrix, a methodology which significantly reduces the viable cell recovery count.[48]

Some limitations to sortase's industrial potential have been identified. The reversibility of sortase cross-linking does risk the reaction not proceeding to completion, even in the presence of significant quantities of enzyme, resulting in unfavourable material properties (altering hardness, and gel fraction).[39,49] To mitigate this problem, it is necessary to conduct SrtA catalysed cross-linking reactions with the poly(glycine) component in excess of that of the component bearing the sorting signal. This reversibility, however, has recently been successfully exploited in the development of a tuneable hydrogel, which proved a viable cell culture system for human mesenchymal stem cells and pancreatic cancer cells. SrtA cross-linked PEG-peptide hydrogels were shown to undergo multiple cycles of gel softening and stiffening, and ultimately complete dissolution through the addition of further SrtA and a soluble glycine substrate (Figure 4).[49]

A further application of sortase cross-linking can be seen in the development of the sortase-mediated transpeptidation or 'Sortagging' approach for the site-specific labelling of proteins with small fluorescent probes. This versatile method can be applied to achieve site-specific protein labelling *in vitro* and on the surface of living cells.[50] Given that substrate specificity can be achieved in such systems through the use of different sortase family members, which recognise alternative sorting pentapeptides, for example, SrtA, LPXTG; SrtB, NP(QK)TN; SrtC, (I/L)(P/A)XTG; SrtD, LPNTA; and SrtE: LAXTG,[35] there is considerable scope for the development of orthogonal sortase-based cross-linking systems. This would enable greater complexity of structure, property and function in biopolymer constructs.

4 | LACCASE AND PEROXIDASE—CROSS-LINKING GOES METAL

Laccases (EC 1.10.3.2) are copper-dependent enzymes that catalyse single electron oxidation reactions and are commonly used for biopolymer cross-linking in organismal biochemistry. They play key roles in the formation and degradation of lignin,[51] are the principle enzymes involved in insect cuticle hardening,[52] and contribute to the production of melanin pigments in fungi (Table 1).[53] Peroxidases (EC 1.11.1.7) are a related and diverse sub-group of the oxidoreductases that catalyse the decomposition of hydrogen peroxide yielding water and molecular oxygen. Their catalytic mechanism involves the abstraction of single electrons from substrate molecules, in tandem with the reduction of hydrogen peroxide to water.[54] Free radicals produced via this route may then participate in additional downstream reactions, including the cross-linking of biopolymers such as lignin.[55]

Although laccases and peroxidases will not be discussed in detail in this review, their value to biopolymer synthesis is important to note. Laccases for example have been used in the preparation of

TABLE 2 Applications of enzyme mediated cross-linking of proteins, non-proteinogenic polymers (highlighted in grey), and small molecules

	Enzyme	Application(s)	Ref(s)
Food			
Food additives and processing	Tyrosinase	Production of phenolic hydroxyl groups for use as food additives such as theaflavins for black tea. Synthesis of secondary polyphenols for food processing. Cross-linking of pea-protein and pea-zein complexes in the stabilisation of emulsions.	[94–97]
	Laccase	Cross-linking of whey protein isolates to enhance emulsion stability.	[98]
Meat	Tyrosinase	Cross-linking of meat proteins in gelation to alter textural and binding properties of meat products.	[99,100]
	Laccase	Cross-linking of myofibril protein to improve gelation effects of chicken proteins.	[101]
	Transglutaminase	Cross-links meat proteins for restructuration to improve the solubility, water-holding capacity and thermal stability of the proteins. Cross-linking of caseinate which can act as a glue to bind meat, eliminating the need for sodium chloride or phosphate addition.	[15,25]
Fish	Transglutaminase	Cross-linking of caseinate to harden fish protein and produce surimi. Binding of a whey protein-based coating to Spanish mackerel for improved preservation of the fish.	[26,102–104]
Dairy	Transglutaminase	Cross-linking of milk casein to a heat-resistant firm gel for milk, yoghurt, and low-fat dairy products.	[15,105–107]
	Tyrosinase	Cross-linking of milk casein to produce yoghurt and cheese.	[108]
Tofu	Transglutaminase	Cross-linking of soybean proteins resulting in coagulation to give tofu a smooth texture when prepared with techniques designed to prolong shelf-life, e.g., high temperature sterilisation.	[15,109]
Noodles and pasta	Transglutaminase	Cross-linking of gluten proteins, increasing molecular weight and allowing low-grade flour to retain the texture of higher grade flour when cooked and processed.	[110,111]
Cereals	Tyrosinase	Polymerisation of gliadin for gluten production which improves the volume and crumb of breads. Also improves texture of gluten free oat bread by cross-linking oat globulins.	[96,112,113]
Sugar beet	Laccase	Cross-linking of fibrex to produce edible gels with higher water holding capacity, better swelling in saliva and heat resistance compared to non-cross-linked fibrex. These could be used to manufacture vegan, halal and kosher foods, as a replacement for gelatin.	[114]
Wheat bran	Laccase	Cross-linking of arabinoxylans to produce a gelatin alternative for the manufacture of vegan, halal and kosher foods.	[114]
Unwanted by-products from production	Tyrosinase	Conversion of the by-products of food processing to environmentally favourable products with functional characteristics, e.g., the conjugation of milk proteins (casein) with chitosan to create biodegradable and environmentally friendly non-food bioproducts.	[71]
Allergy reduction	Transglutaminase	Cross-linked peanut hydrolysates (hydrolysed with papain, ficin or bromelain) reduced peanut allergenicity while retaining functional properties usually lost with hydrolysis.	[115]
	Tyrosinase	Cross-linked fish parvalbumin shows a reduced amount of IgG bound compared to non-cross-linked parvalbumin, so reducing allergenicity.	[116]
Textiles			
Wool	Transglutaminase	Cross-linking casein, gelatin, keratin, and silk proteins to wool for increased tensile strength and smoothness of the fabric.	[6,28,117,118]
	Tyrosinase	Activation of tyrosine residues to attach biopolymers such as collagen to create textiles that can be used as a substratum to proliferate micro-organisms.	[73]

TABLE 2 (Continued)

	Enzyme	Application(s)	Ref(s)
Leather	Transglutaminase	Cross-linking of gelatin and casein for the improvement of grain smoothness and fullness, and for improvement of resistance against washing damage. Used as a filler for voids in animal hide.	[6,119]
Cosmetics			
Bonding agent	Transglutaminase	Bonds amine groups in active ingredients (present in cosmetics/sunscreen) to glutamine groups on the surface of skin, hair and nails.	[120]
Self-tanner	Tyrosinase	Stimulation of melanogenesis in self-tanning creams containing mixtures of acetyl tyrosine and chaste berry extracts. Increases skin melanogenesis through increasing the bioavailability of tyrosine in skin by creating more soluble tyrosine derivatives.	[121–123]
Biological materials/drug delivery			
Drug delivery	Tyrosinase	Activation of prodrugs at melanomas. Production of L-DOPA in immunoassays and antibody microarrays. Biosensor to detect L-tyrosine levels in organisms.	[124–126]
	Peroxidases, commonly HRP	Cross-linking of aromatic groups resulting in functionalised polyaspartic acid to improve drug delivery. Cross-linking of silk sericin to PEG dimethacrylate to generate hydrogels which sustain drug release.	[127–136]
	Transglutaminase	Cross-linking of PEG-peptide hydrogels for disassembly by cell-secreted proteins which could result in location-based drug delivery.	[48,137–141]
	Sortase A	Conjugation of antibodies (modified heavy IgH and light IgL chains) to anti-tumor drugs to provide defined drug to antibody ratios unlike chemical methods. Conjugation of PEG to cytokines to improve drug half-life in therapeutic applications.	[142,143]
Tissue engineering and ECM construction	Kinase/phosphatase	Controlled phosphorylation and dephosphorylation of a pentapeptidic hydrogelator to form a supramolecular hydrogel in the presence of adenosine triphosphates via the self-assembly of nanofibers as a result of the enzyme action.	[144]
	Sortase A	Cross-linking of hyaluronan-based synthetic ECM. Cross-linking of poly(2-ethyl-2-oxazoline)-peptide conjugates to generate hydrogels.	[39,145,146]
	Peroxidase, commonly HRP	Cross-linking of functionalised polyaspartic acid for tissue and wound healing. Cross-linking of chitosan derivatives, hyaluronic acid-tyramine and alginate-phenol tyramine for tissue engineering. Cross-linking of hyaluronic acid-tyramine, chitosan-glycolic acid conjugates modified with phloretic acid, dextran-tyramine conjugates, dextran-hyaluronic acid conjugates and dextran-heparin used to repair cartilage tissues. Cross-linking of fish gelatin to produce hydrogels capable of supporting human dermal fibroblast cell adherence and proliferation with controllable properties. Cross-linking of silk to generate versatile hydrogel microfibers.	[127,128,130–134,136,147–154]
	Transglutaminase	Cross-linking of lysine/glutamine containing hydrogels for in situ gelation. Cross-linking of PEG-peptide and fibrin hydrogels to repair cartilage tissue. Cross-linking of hyaluronan hydrogels as a synthetic ECM. Cross-linking of PEG-peptide hydrogels used to make smart implants. Cross-linking of gelatin and chitosan to generate scaffolds.	[138,140,141,155–164]
	Tyrosinase	Cross-linking of silk fibroin and chitosan to produce polymeric scaffolds with novel physiochemical properties to its constituent parts. Potential application as wound dressing because of non-toxicity. Cross-linking of silk and gelatin for bioprinting skin models.	[165,166]

(Continues)

TABLE 2 (Continued)

	Enzyme	Application(s)	Ref(s)
Tissue adhesives	Transglutaminase	Cross-linking of PEG-peptides for surgical tissue glues.	[163,167]
	Tyrosinase	Production of dopamine-chitosan conjugated bio-polymer systems which confer novel water-resistant adhesive properties. Strength can be modulated by altering chitosan/gelatin ratios. Tyrosinase modification resulted in an improvement in the adhesive abilities of a soyabean protein-based adhesive. Cross-linking of epigallocatechin gallate conjugated hyaluronic acids and tyramine conjugated hyaluronic acids to form an anti-inflammatory and adhesive hydrogel.	[168–170]
Cell culture	Thermolysin	Aids in constructing a scaffold for cells based on a Fmoc-(Phe)3 hydrogel.	[171]
	Transglutaminase	Cross-linking of fibrin to produce fibrin gels which assist with angiogenesis and neurite extension by supporting endothelial cells and encouraging proliferation. Tethers PEG-based cell-adhesion ligands.	[48,158–160,164,172]
	Phosphopantetheinyl transferase	Cross-linking of PEG-based hydrogels using multi-arm PEG macromers end-functionalised with CoA.	[173]
Biomineralisation	Phosphatase	Construction of biomineralisation scaffolds from supramolecular tyrosine-phosphate-based hydrogels.	[174]
Wound dressings	Tyrosinase	Cross-links gelatin-chitosan conjugated biopolymers for applications such as skin substitutes and wound dressings.	[168,175]
Protein immobilisation	Tyrosinase	Production of a chitosan-Kcoil scaffold used as a protein immobilisation technique.	[176]
	Sortase A	Binding of enzymes (biocatalysts) to solid surfaces including beads, agarose and glass.	[177]
Film fabrication	Tyrosinase	Catalysis of gelatin-chitosan conjugation for use as scaffold for tissue engineering.	[155,178]
Building materials			
Wood	Laccase	Production of fibreboards by the oxidation of wood fibres and cross-linking of lignin.	[56]
Environmental testing			
Biosensors	Tyrosinase	Detect water and soil levels of toxic waste phenols by polymerizing industrial phenols produced as industry byproducts, e.g., in synthetic polymer production, petrochemical, wood-pulp and dye production. Detection of phenol level in beer. Biosensor for analysis of ascorbic, benzoic, gallic and kojic acids.	[178–182]
	Transglutaminase	Construction of microfluidic biosensor systems from gelatin.	[183]
Others			
Gelation model	Transglutaminase	Gelation of multi-arm comb PEG.	[184]
	Tyrosinase	Cross-linking in hydrogel formation between gelatin containing collagen, casein or albumin components.	[185,186]
Fuel cells	Sortase	Ligation of a streptavidin tag to an azido-containing tri-glycine to generate a hydrogel which covers an electrode with the ability to immobilise glucose dehydrogenase in a glucose/O_2 fuel cell.	[187]
Glue	Tyrosinase	Expression of tyrosinase in biofilm-based adhesives improved adhesive properties through production of DOPA-quinones which subsequently cross-linked.	[188]

Abbreviations: CoA, coenzyme A; ECM, extracellular matrix; HRP, horseradish peroxidase; PEG, poly(ethylene glycol).

fibreboard, exploiting the enzyme's capacity to cross-link wood fibres to lignin.[56] Bioactive coatings containing immobilised laccase have also been made, affording a route to the preparation of biocatalytically active materials with use in cross-linking for material bonding applications.[57] Similarly, peroxidases (in particular horseradish peroxidase [HRP]) have also been used for polymer cross-linking. Hydrogels formed via HRP catalysed cross-linking have proven to be an effective medium to support mammalian cell culture, due to their rapid and

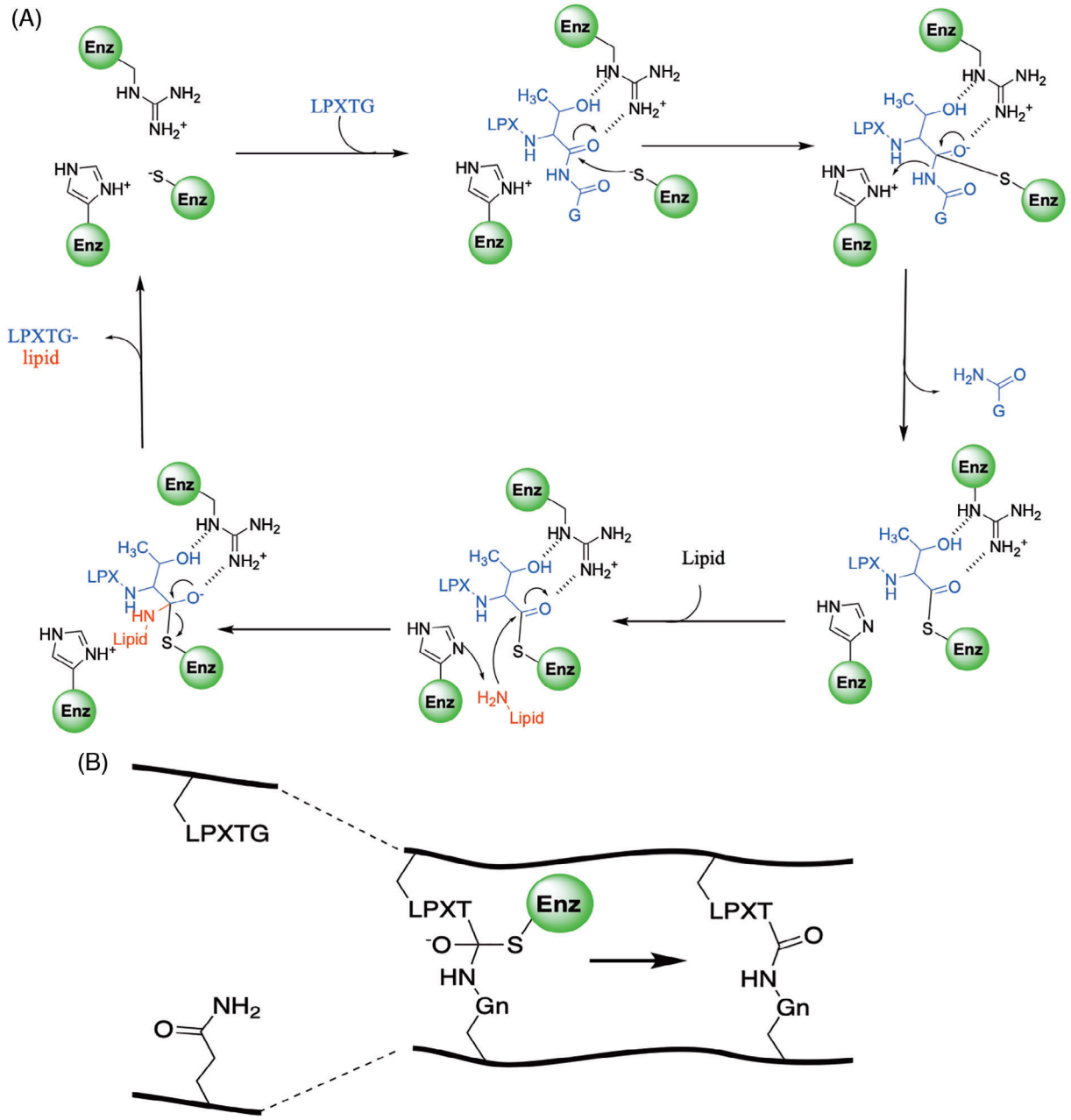

FIGURE 3 A, Catalytic mechanism of the transpeptidase sortase A.[191] SrtA catalyses cleavage of the peptide bond between the threonine and glycine residues within the substrate sorting signal, yielding a thioester intermediate within the enzyme active site. This intermediate undergoes nucleophilic attack by the N-terminal glycine of the secondary substrate, resulting in the formation of an amide bond between the threonine of substrate 1 and the glycine of substrate 2. B, Schematic representation of sortase A catalysed polymer cross-linking

tuneable gelation rates under physiologically relevant conditions.[58] Due to the respective nature of the oxidants used by HRP and laccase, HRP has been shown to have faster gelation rates than laccase in the cross-linking of tyrosine-modified PVA hydrogels.[59]

5 | LYSYL OXIDASE—THE ECM RELOADED

Lysyl oxidases (LOXs) (EC 1.4.3.13) are extracellular copper containing metalloenzymes that are widely distributed in animals, bacteria and archaea.[60–62] To date, mammalian LOX has been the most intensively studied form of the enzyme, due to its key role in remodelling the ECM. Mammalian LOX catalyses the final stages of elastin and collagen cross-linking within the ECM, via a mechanism that involves the oxidative deamination of lysine and hydroxylysine side chains on collagen and elastin precursors to produce reactive allysine groups.[61,63] These reactive aldehyde groups spontaneously condense with vicinal peptidyl lysine, hydroxylysine or allysine residues to produce covalently cross-linked products (Figure 5). The extent of covalent cross-linking contributes to the tensile and elastic strength of fibrous proteins such as collagen and elastic proteins such as elastin. All members of the LOX enzyme family possess a highly

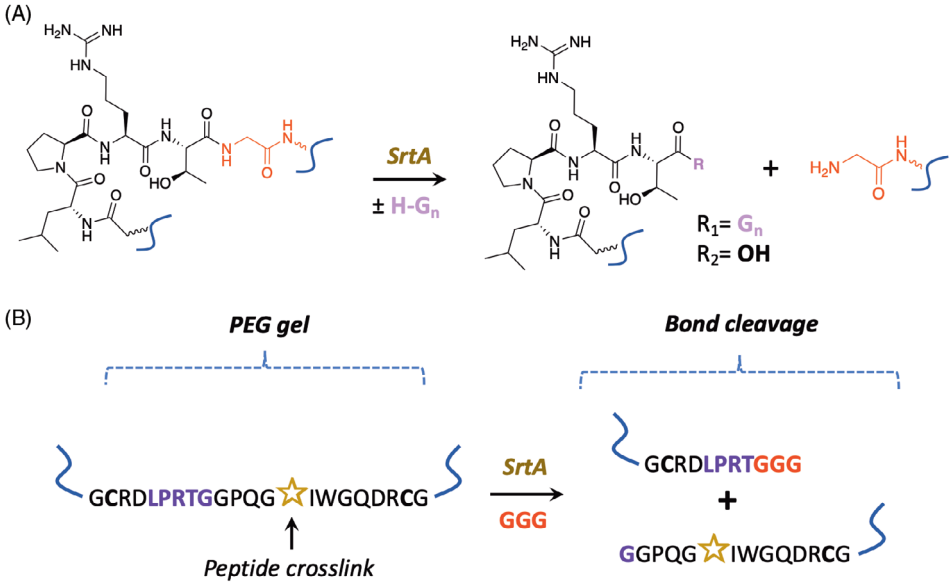

FIGURE 4 SrtA-mediated bond cleavage and hydrogel dissolution mechanisms. A,Schematic representation of the SrtA-mediated peptide cleavage method developed by Arkenberg *et al*.[49] Hydrogels could be readily degraded through the addition of SrtA and soluble glycine substrates (e.g., glycinamide). B, SrtA in combination with a soluble GGG tripeptide facilitates a transpeptidase reaction that functionally severs PEG hydrogel crosslinks as reported by Valdez *et al*.[47] Purple = SrtA substrate, red = soluble GGG tripeptide, orange star = matrix metalloproteinase sensitive sequence for cell-mediated remodelling

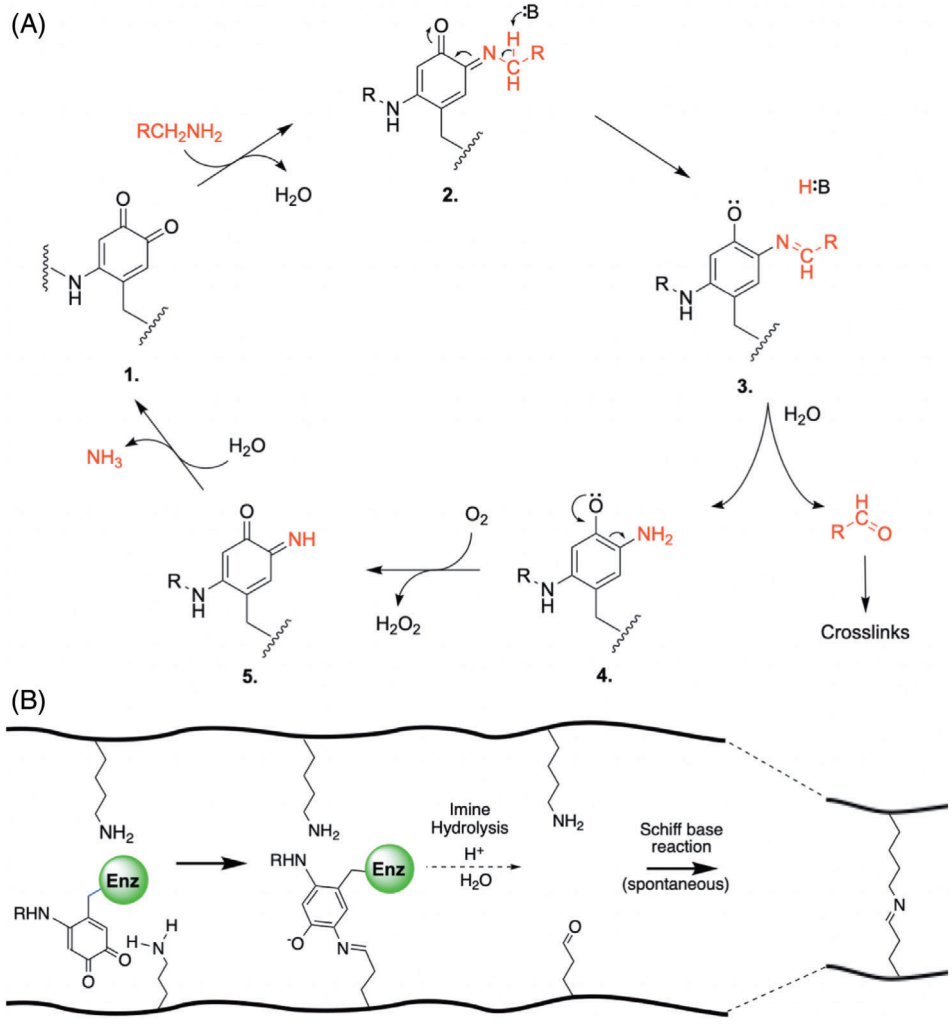

FIGURE 5 A,Catalytic cycle of lysyl oxidase (LOX).[192] The enzyme catalyses the conversion of lysine residues to α-aminoadipidic-δ-semialdehydes (allysines). During the oxidation reaction, the lysine ε-amine is first converted to a Schiff base via a reaction which is dependent on the cofactor lysyl tyrosyl quinone (LTQ). Rate-limiting removal of the ε-proton yields an imine intermediate, with subsequent imine hydrolysis leading to liberation of the aldehyde product. B, Schematic representation of LOX catalysed polymer cross-linking

conserved C terminal domain, which houses the enzyme active site. The presence of bound copper (II) in this site is required for the formation of a lysyl tyrosyl quinone cofactor, whose necessity for catalysis remains the subject of some debate.[64] Although mammalian LOX has significant potential for use as a biocatalytic cross-linker, it has been significantly underexploited to date, due to recurring issues in the preparation of high purity, homogeneous protein, in either native or recombinant forms.

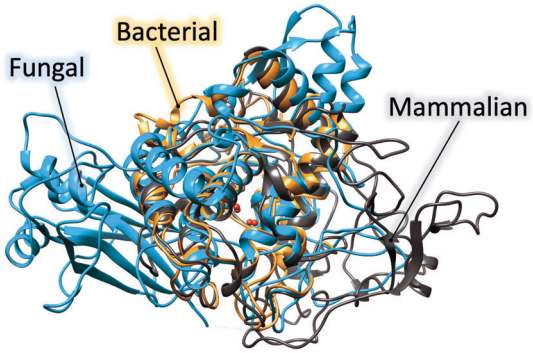

FIGURE 6 Superposition of the crystal structures of fungal (blue, PDB 5M6B), mammalian (grey, PDB 5M8L) and bacterial (orange, PDB 3NMB) tyrosinase. The core protein fold which houses the copper (red spheres) containing enzyme active site is conserved across multiple species

6 | TYROSINASE—PUTTING THE 'OH' INTO BIOCATALYSIS

Tyrosinase (EC 1.14.18.1), or polyphenol oxidase, is a di-copper containing metalloenzyme found in both prokaryotes and eukaryotes.[65,66] Tyrosinases from plants, bacteria, fungi and humans show differences in structure, activation, localisation and oligomeric state (Figure 6). Although tyrosinase performs similar reactions across phyla, it has varying physiological roles in different organisms (Table 1). In animals, tyrosinase catalyses the initial steps of melanin formation from tyrosine.[67] In plants, tyrosinase catalyses the oxidation of phenolic compounds in fruits to quinones causing an unpleasant odour or taste beyond the point of human appeal, and is responsible for undesirable enzymatic browning that occurs post-harvest, or as a consequence of bruising.[68] In insects, tyrosinase contributes to melanisation and the immune response, and plays a critical role in sclerotisation as a biocatalytic cross-linker.[69]

Tyrosinase is capable of performing two distinct reactions once activated through oxygen binding. The first is a monophenolase reaction cycle where activated tyrosinase catalyses the conversion of phenols such as tyrosine to *o*-diphenol intermediates that are subsequently oxidised to *o*-quinone products (Figure 7). This oxidative process leaves tyrosinase in a reduced state, from which it is reactivated by oxidation by molecular oxygen back to a catalytically competent state. The second is diphenolase activity, where activated tyrosinase catalyses the conversion of diphenols (e.g., L-DOPA) to *o*-quinones (Figure 7). Following a single turn-over event, the enzyme remains in a resting reduced state, retaining the capacity to catalyse a second diphenolase reaction to yield an additional *o*-quinone product. Finally, the reduced enzyme is reoxidised by molecular oxygen.[70] The *o*-quinone product of these reactions will react avidly and spontaneously with large or multifunctional nucleophiles to generate covalent cross-links.

Tyrosinase shows considerable promise for use in industrial processes, including in applications as diverse as food preparation, textile and cosmetic manufacture, drug formulation and delivery, and in biosensing (Table 2). It has been used in waste product processing in the dairy industry for the conversion of the phosphoprotein casein into high-value non-food polymers. Casein contains 6% to 8% tyrosine and can be readily cross-linked to the amine-functionalised polysaccharide chitosan (a by-product of shellfish processing) to generate cross-linked polymeric materials.[71] It has been shown that tyrosinase catalysed reactions between chitosan (0.32%) and casein (0.5%) generate cross-linked polymers with novel viscoelastic properties, which can be tuned by adjusting the ratio of the polymer substrates used.[72] In the textile industry, tyrosinase has also been shown to cross-link tyrosine residues in wool and silk fibroin to other biopolymers such as collagen and elastin.[73] This creates a mechanically strong coated material that has been shown to have bactericidal and fungicidal properties effective against bacteria such as *S. aureus* and *K. pneumoniae*.[73] For this reason, such materials have been employed as components of wound dressings for use in hospital settings.

7 | BENEFITS, CHALLENGES AND LIMITATIONS OF ENZYME CATALYSED CROSS-LINKING

Many industrial sectors are dependent on thermoset cross-linked polymers made using cheap, well-established chemical cross-linking methods. However, in the same way that the chemical industry is 'greening' its large-scale processes, often by adopting biocatalysis and biotransformation, the case for the broader adoption of enzymes as cross-linking agents is becoming an increasingly compelling one. Enzymes offer a catalytic methodology to induce cross-linking reactions with reaction types both mirroring and distinct from existing chemical technology. In contrast to chemical methods, their chemo- and stereo-selectivities are inherent characteristics, enabling precise cross-linking reactions to be performed reproducibly and at scale. Despite this, there are barriers to adoption and the use of enzymes as cross-linkers in industrial scale manufacturing processes remains the exception rather than the norm.

Despite being able to produce stocks of enzymes using a suitable host, in contrast to the resource implications of many chemical reagents, sourcing enzymes of sufficient purity and in sufficient quantities, either from natural sources or in recombinant form, remains a challenge. Low enzyme yields, restrictive storage conditions, and the limited shelf-life of many enzymes adds to these challenges, and such supply issues have constricted the use of enzymes to small to medium scale processes. For example, the transglutaminase factor XIII has been widely used as a cross-linker in hydrogel fabrication; however, this enzyme is only moderately stable at room temperature and the kinetics of factor XIII gelation rapidly reach a plateau where further addition of enzyme does not increase gelation rate.[39]

Encouragingly, the past decade has witnessed significant improvements in recombinant protein production technologies that go some way towards circumventing the issues of scaling up production. In parallel, major advances in gene synthesis capability and associated cost reductions have made biocatalyst discovery and

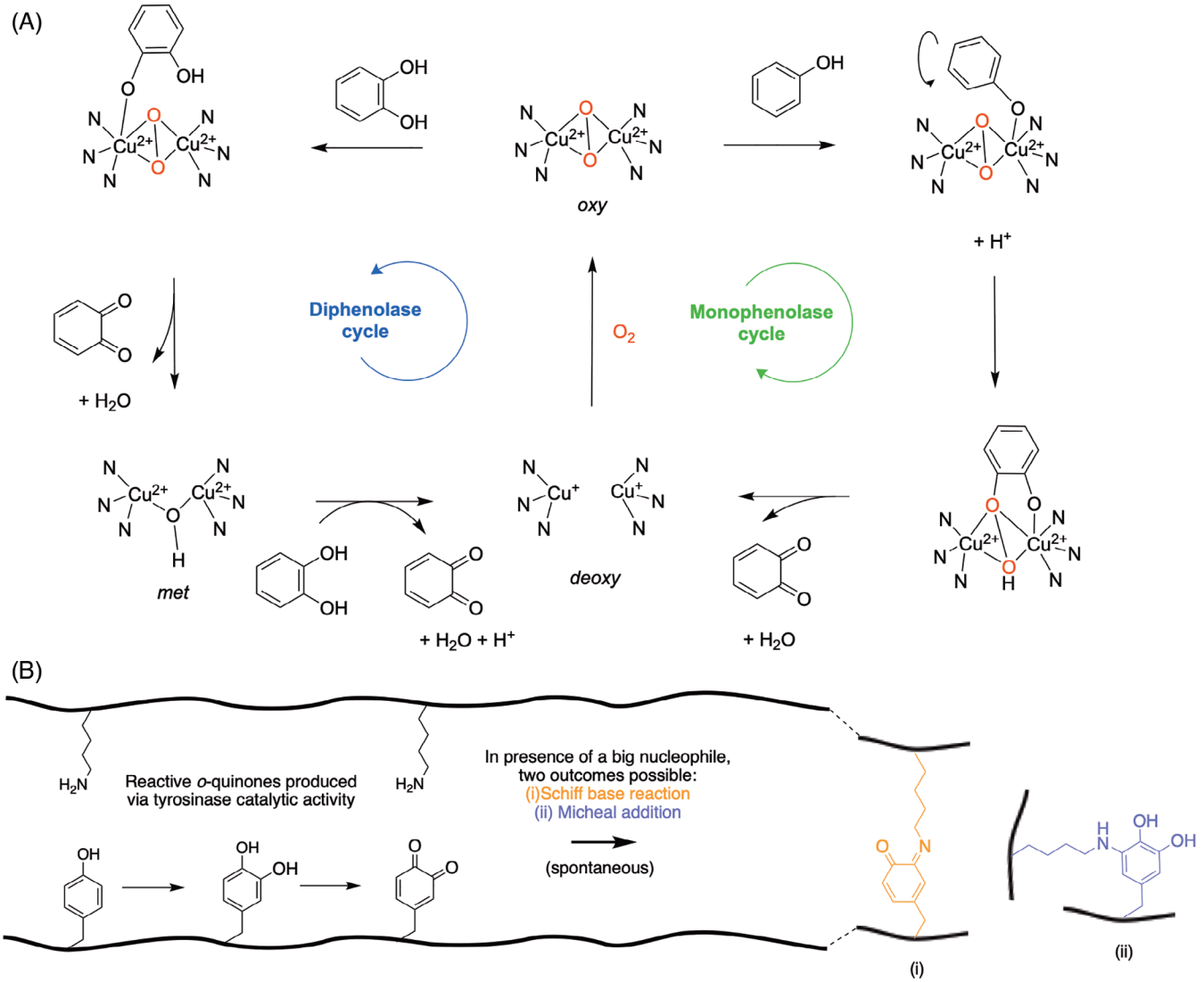

FIGURE 7 A, Catalytic cycle of tyrosinase.[193] The enzyme is capable of performing two distinct reactions once activated through oxygen binding. The first is a monophenolase reaction cycle where activated tyrosinase catalyses the conversion of phenols such as tyrosine to *o*-diphenol intermediates, which are subsequently oxidised to *o*-quinone products. Resulting reduced tyrosinase may then be reactivated by oxidation by molecular oxygen. The second is a diphenolase reaction, where activated tyrosinase catalyses the conversion of diphenols (e.g., L-DOPA) to *o*-quinones. B, Schematic representation of tyrosinase catalysed polymer cross-linking, with two potential cross-linked products shown

production in heterologous hosts a widely exploited route to method development. Protein engineering has also matured as a discipline, providing access to optimised variants of natural proteins with improved kinetic performance and stability. For example, engineered SrtA enzymes have been shown to retain activity for >48 hours at room temperature, and up to 140-fold improvement in coupling activity, and exhibiting linear reaction kinetics with respect to enzyme concentration.[74] SrtA can cross-link polymers via an analogous mechanism to factor XIII, through functionalisation with vinylsulfones followed by conjugation to thiol containing peptides via Michael addition, so the suitability of this enzyme for manufacture at scale along with its favourable kinetics and stability should encourage the wider adoption of this biocatalyst for cross-linking applications. One drawback of using bacterial cell culture for enzyme production is the potential for endotoxin contamination; however, the use of endotoxin removal resin has been shown to be effective in reducing the concentration of this contaminant to below FDA accepted levels.[39]

Many applications of enzyme catalysed cross-linking capitalise on the inherent biocompatibility of enzymes. For example, it is widely accepted that enzyme catalysed cross-linking approaches for the fabrication of synthetic hydrogels are preferable to the use of chemical cross-linkers.[3,75] This is due to a combination of factors including their reduced toxicity and ability to cross-link constituent polymers under physiological conditions. Similarly, in the food industry, transglutaminase has found widespread use as a cross-linker in the preparation of foodstuffs (Table 2), enabled by its acceptable safety profile in humans and animals. It is important, however, to consider the deleterious consequences of off-target activities catalysed by enzymes and any potential toxic by-products that may be generated by their use. For example, peroxidases are notorious for their substrate

promiscuity, which has limited their usefulness in hydrogel preparation for mammalian cell culture.

Considering the currently characterised enzyme classes which can perform reactions suitable for cross-linking and their broad substrate tolerances, there is certainly no shortage of options available for cross-linking applications. The ongoing process of enzyme discovery will inevitably lead to new enzymatic process to exploit, and greater access to the chemical toolbox. The ability of enzymes to recognise and activate specific functional groups displayed on a variety of chemically distinct polymers, not all of which are strictly biopolymers, allows for both natural and non-natural polymeric substrates to be fused, presenting an almost endless array of possibilities for starting materials and end products.[76] Roberts *et al.* have shown that commodity polymers such as dextran, HA, PEG and PVA can be appended with phenolic substituents (tyramine, or hydroxyphenylpropionic acid, or 4-hydroxyphenyl acetic acid) and then cross-linked by the action of oxidative enzymes such as tyrosinase and laccase.[59] Similarly, biohybrid polymer conjugates can be readily generated using enzymatic cross-linking, as illustrated by the tyrosinase directed assembly of silk-gelatin hydrogels for use in tissue engineering and cell delivery applications.[77]

Of the enzyme classes mentioned in this review, transglutaminases and peroxidases have the lowest specificity for cross-linking components discriminating solely on the basis of amino acid or functional group identity and accessibility. For this reason, they may be best suited to generic cross-linking applications including the preparation of bulk materials where turnover rather than selectivity is of paramount importance. In contrast, enzymes such as sortases are highly substrate selective and are thus better suited to bespoke cross-linking applications. It may be that a hybrid multi-enzyme approach may instead leverage the respective advantages of multiple biocatalytic cross-linkers within a single use case. This approach was elegantly demonstrated by Arkenberg and Lin, who used SrtA in combination with tyrosinase to produce PEG-peptide hydrogels with the ability to mimic ECM stiffening.[38] Initial cross-linking of the PEG polymers was performed using SrtA to establish a hydrogel network, which was subsequently stiffened by the introduction of additional cross-links using tyrosinase. The secondary rigidification of the hydrogel scaffold closely mimics effects observed in the ECM during cancer progression and wound healing.[78–80]

There are of course potential pitfalls which must be avoided when deploying enzymes in cross-linking applications. The reliance of many enzymes such as tyrosinase and LOX on co-factors limits their scope and usefulness. In some cases this has been overcome by identifying functional co-factor-independent homologues such as calcium-independent microbially derived transglutaminases, or through the use of mutagenesis as for SrtA.[35,81] It should be noted, however, that these are not approaches that can be universally applied to all enzymes of all classes and the ease by which this can be achieved will be to a large extent dictated by the precise role of the cofactor in question, that is, in maintaining structural integrity or in catalysis itself. In addition, enzymes must bind and appropriately orient substrate molecules within their active sites for catalysis to proceed, something that will be demanding for polymeric materials with limited freedom of movement and significant steric bulk. In such circumstances where target substrate functional groups are occluded or inaccessible this would preclude the use of enzymatic cross-linking of any kind. For example, HRP has been used to generate networks of cross-linked bovine R-lactalbumin proteins, but only in instances where the calcium co-factor of R-lactalbumin is first removed, reducing the rigidity of the polypeptide chain and enabling access to addressable amino acid side chains within the protein.[82] It should be remembered, however, that synthetic polymers are generally more dynamically heterogeneous than proteins and contain a higher density of activatable functional groups. As such they are, perhaps counterintuitively, better suited for use in applications that incorporate biocatalytic cross-linking.

8 | INDUSTRIAL ADOPTION AND FUTURE PROSPECTS—WHO IS BETTER WHO IS BEST

Enzymes are now being increasingly viewed as a viable alternative to chemical cross-linking approaches in some fields. The breadth of industrial process that use biocatalytic cross-linking reactions is already significant and continues to grow (Table 2). The food industry in particular has exploited biocatalytic cross-linking to great effect, adopting transglutaminase in food preparation and processing, and tyrosinase in texturising agents. The textile industry has also been a major promoter of enzymatic cross-linking, where targeted transglutaminase treatment is used to promote the wettability, softness and tensile strength of fabrics, and to promote colour fastness. The biomedical sector has a requirement for soft materials which mimic the structural properties of the ECM to facilitate tissue engineering and the formulation of drug delivery vehicles. Enzyme catalysed cross-linking is having major impact in this area by offering the ability to meld biomaterials and chemical alternatives using non-toxic chemistries. By contrast, there are very few examples of the use of biocatalytic cross-linking in the production of hard materials, possibly due to the intermolecular constraints which provide the strength while hampering access to all but the outermost layers. Whether bulk modification of hard materials or the creation of hard materials is a step too far is debatable, but the use of cross-linking enzymes to alter surface properties of such materials is a clear opportunity.

While “small molecule” chemistry, as epitomised by medicinal chemistry, both in its discovery and scale up activities, has been relatively swift to adopt biocatalysis to provide stereochemical and regiochemical precision, polymer chemistry has been significantly slower to delve into the enzyme discovery toolbox, certainly with respect to cross-linking reactions. This may be in part due to the nature of the polymers themselves, or a perceived deficiency in available enzyme classes that can offer a suitable breadth of reaction types. Despite this, we hope to have demonstrated herein that there are currently several vibrant lines of enquiry exploring the potential of enzymes as cross-linking agents, with this area now primed for exponential growth in the coming years.

ACKNOWLEDGEMENTS

The authors thank the BBSRC (BB/L01386X/1, BB/T001968/1, BB/M012107/1 and BB/M025624/1) and EPSRC (EP/R020957/1) for grant funding. R. M. A. M. is supported by an EPSRC funded PhD studentship (EP/L016494/1) administered by the Synthetic Biology Centre for Doctoral Training. G. J. P. is supported by a University of Bristol scholarship for postgraduate study.

CONFLICT OF INTEREST

The authors declare no competing interests.

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How to cite this article: Maddock RMA, Pollard GJ, Moreau NG, Perry JJ, Race PR. Enzyme-catalysed polymer cross-linking: Biocatalytic tools for chemical biology, materials science and beyond. *Biopolymers*. 2020;111:e23390. https://doi.org/10.1002/bip.23390