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REVIEW

Enzymatic Methods for Assembling and Modifying Hydrogel Biomaterials

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Abstract

Purpose Enzymatic reactions offer many advantages for hydrogel synthesis and modification, due to their gentle reaction conditions, biocompatibility, and diversity of substrates.

Methods In this review, we examine the current body of literature through databases such as Google Scholar, PubMed, and Web of Science.

Results Various enzyme classes have been utilized for hydrogel assembly and disassembly, including transglutaminases, oxidoreductases, transpeptidases, and proteinases. The enzymatic substrates can be readily included in peptide precursors and/or appended onto synthetic polymers. We discuss the benefits and limitations of each system, with a focus on ease of use/synthesis, accessibility, and financial considerations.

Conclusion Enzymes are frequently utilized to modify both natural and synthetic biomaterials. For developing more advanced, stimuli-responsive platforms, "biologically invisible" enzymes such as sortases should be leveraged to not interfere with native processes and/or the mammalian proteome.

Lay Summary Enzymes, proteins that act as biological catalysts, are an important tool for making and breaking down hydrogels, or water-swollen polymeric networks, for various biomedical applications. In particular, these techniques have seen great usage for modeling the tissue environment for lab-based assays.

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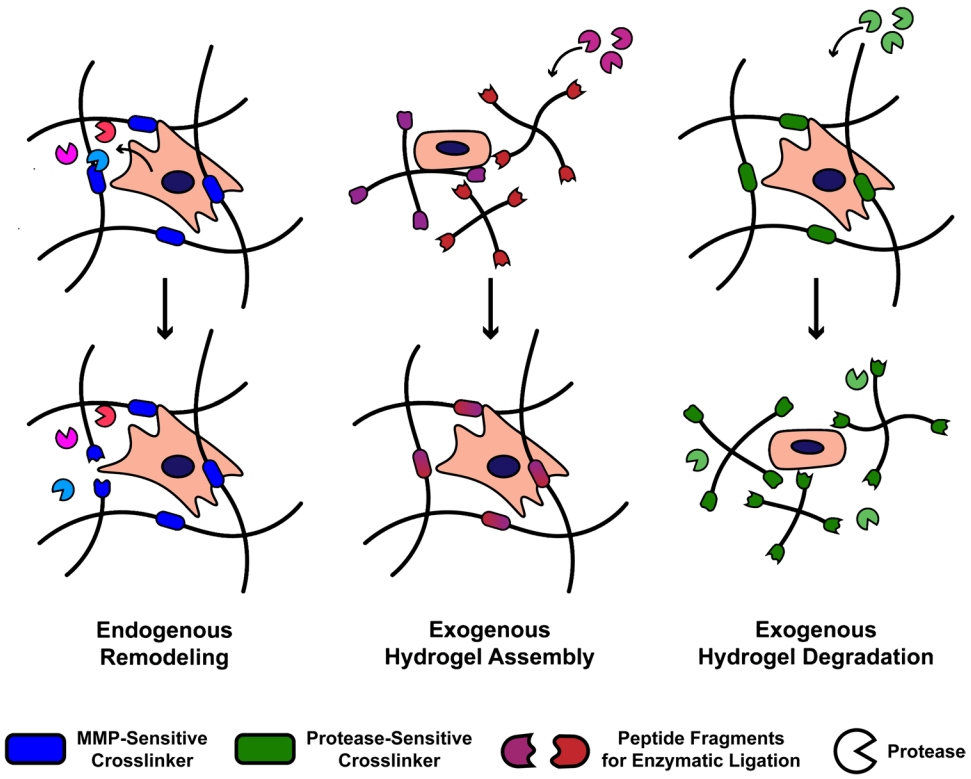
Graphical Abstract



Keywords Hydrogel · Stimuli-responsive · Enzymes

Introduction

Enzymes—proteins that act as biological catalysts—surround us in our daily lives. Their role in regulating and accelerating bioreactions is indispensable; as of 2024, over 8000 characterized enzymes have been identified and subdivided into 7 main classes (i.e., oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases), driving numerous critical reactions in cellular metabolism and function [1, 2]. Enzymes are globular proteins with a substrate-binding active site (usually a small polypeptide domain ranging from 1 to 10 amino acids whose distinct shape and charge profile afford high substrate specificity and catalytic activity [3]. Unsurprisingly, enzymatic biocatalysis has seen widespread use in many distinct commercial and research fields, with applications ranging from industrial small molecule chemical synthesis to designer therapeutic agents.

In bioconjugation and biomaterials research, the latter heavily reliant on the former, use of enzyme-based strategies to affix or cleave moieties to/from macromolecular structures has gained popularity owing to their efficacy, specificity, and mild reaction conditions. Although the term "biomaterial" encompasses a wide variety of materials that interface with biological systems, this Review focuses on hydrogel biomaterials—water-swollen polymeric networks that can act as three-dimensional (3D) extracellular matrix (ECM) mimics or drug-eluting reservoirs. These polymeric systems, derived from natural and/or synthetic precursors, can be readily endowed with stimuli-responsivity [4]. While other stimuli, such as light, temperature, or pH, are commonly employed for hydrogel biomaterial synthesis or modification [5–7], the use of enzymes offers unique advantages over many other strategies. In particular, enzymes (1) are gentle and biocompatible, (2) can be readily engineered via mutational screens to recognize ligands with high specificity, (3) do not suffer from attenuation, such as light, and thus, could be utilized in vivo, (4) offer kinetic control over the reaction by changing concentrations or modulating enzymatic activity, and (5) can be readily obtained via large-scale fermentation. Many of the enzymatic reactions employed in the biomaterials field recognize and act upon short peptide sequences; these peptides are commercially available or amenable to solid-phase synthesis and can be easily incorporated into macromer design via a variety of bioconjugation strategies. Conversely, if complexity is desired, enzymatic

reactions offer a route for multiplexing orthogonal stimuli, either through orthogonal enzymatic recognition sequences or through a combination of other chemistry types. Much of the beauty of enzymatically sensitive systems lies in their intrinsic simplicity, yet opportunity to expand the design space with increasingly bespoke systems.

This Review discusses the current state-of-the-art techniques and enzymes employed for assembly, modulation, and dissolution of hydrogel biomaterials, and offers future perspective for their use in materials science.

Prevalent Enzymatic Reactions in the Hydrogel Field

Tissues in our bodies rely heavily on enzyme-mediated reactions to retain ECM structure and stability. For example, lysyl oxidase (LOX) mediates collagen and elastin assembly by catalyzing the conversion of lysine residues to the aldehyde-containing allysine, which can then react with unmodified lysine residues to form stable crosslinks [8]. This pathway is crucial to tissue development and cellular differentiation; however, it is often dysregulated in many diseases, such as cancer, where upregulation of LOX activity causes tissue stiffening and promotes tumor progression and metastasis [9]. Taking inspiration from nature, "molecular glue"-type enzymes, including thrombin and transglutaminase that are involved in the coagulation cascade, gained early popularity for crosslinking of natural fibrin and synthetic poly(ethylene glycol) (PEG)-based hydrogels [8, 10]. Bacterial-, plant-, and fungal-derived enzymes have also recently gained traction as options for inducing crosslinking and as methods for secondary stiffening of hydrogel networks to mimic disease progression [10].

ECM of native tissues is in a constant state of remodeling during normal development; examples include matrix reorganization during angiogenesis, as well as the creation and dissolution of a clot once the wound has healed. Matrix metalloproteinases (MMPs) are a key group of protease enzymes involved in disassembling the ECM. While natural biopolymer-based hydrogels (e.g., collagen, hyaluronic acid) have intrinsic degradation sites, covalently stabilized synthetic polymer-based hydrogels cannot be readily digested by cellular enzymes; protease-responsive peptides can be included within the network backbone to promote cell-mediated growth and matrix remodeling. The most commonly utilized of such substrates has been the pan-MMP-sensitive peptide sequence GPQG↓IWGQ (where ↓ denotes cleavage site), first reported in 1996 [11]. Since then, significant efforts have been put forth to determine crosslinking peptide sequences sensitive to specific MMPs for tighter microenvironmental control [12–14]. However, these sequences, while degradable by adding exogenous MMPs, collagenase, or trypsin, are typically included for cellular remodeling, as opposed to user-directed modulation of the environment [15]. For those wishing to degrade biomaterials for mechanical modulation or for total gel dissolution, "biologically invisible" enzymes such as those discussed below are recommended [10].

Thrombin and Factor XIIIa

Fibrin gels, prepared with fibrinogen and thrombin—key proteins involved in blood clotting—were among the first natural biomaterials used to prevent bleeding and promote wound healing. Thrombin, which is proteolytically generated by the cleavage of prothrombin by Factor Xa, converts fibrinogen into fibrin by severing the fibrinopeptides A and B, exposing a tripeptide (Gly-Pro-Arg) in the center of the A monomer. This exposed "knob" region can then fit into complementary holes on the C-termini, forming a half-staggered fibril; fibrinopeptide B cleavage is much slower and not necessary for fibril formation but is thought to provide additional structural support [16, 17]. To covalently stabilize the fibrils, factor XIIIa (FXIIIa), a plasma transglutaminase generated from the cleavage of the precursor factor XIII by thrombin in the presence of Ca^{2+}, forms ε-(γ-glutamyl) lysyl isopeptide bonds [16, 18]. This cascade leads to the formation of a fibrous structure that harbors many cellular adhesion cites and binds growth factors; these properties have popularized this self-assembling material for uses in hemostatic glue, drug delivery, and tissue engineering [16, 19]. However, fibrin is susceptible to rapid degradation by cell-secreted plasmin—a desirable trait in normal blood clotting, but a drawback for long-term culture; this can be circumvented by the use of plasmin inhibitors such as aprotinin [19] or by applying these crosslinking cascades to bioinspired synthetic materials.

Towards creating more stable, tougher, and synthetically tunable materials, the Griffith group first pioneered the usage of FXIIIa to crosslink PEG-macromers bearing glutaminamide residues and poly(lysine:phenylalanine) into stable hydrogels on the order of hours (Fig. 1A) [20]. While a successful proof of concept, the relatively slow gelation placed limitations on gel uniformity and cellular applications [7]. However, these studies opened the door for a wave of follow-up research in optimizing substrate sequences to improve kinetics [21, 22]. Since then, multiple studies have used FXIIIa for crosslinking both fully synthetic, composite, and naturally-derived hydrogels for purposes such as vascularized bone mimicking niches and injectable biomaterials [23–25]. Photocaging the ε-amine on the substrate lysine has allowed for spatiotemporal control over FXIIIa-mediated protein immobilization in hydrogels [26, 27].

While FXIIIa has only been used for assembly of hydrogels, its thrombin counterpart, whose native role is to cleave

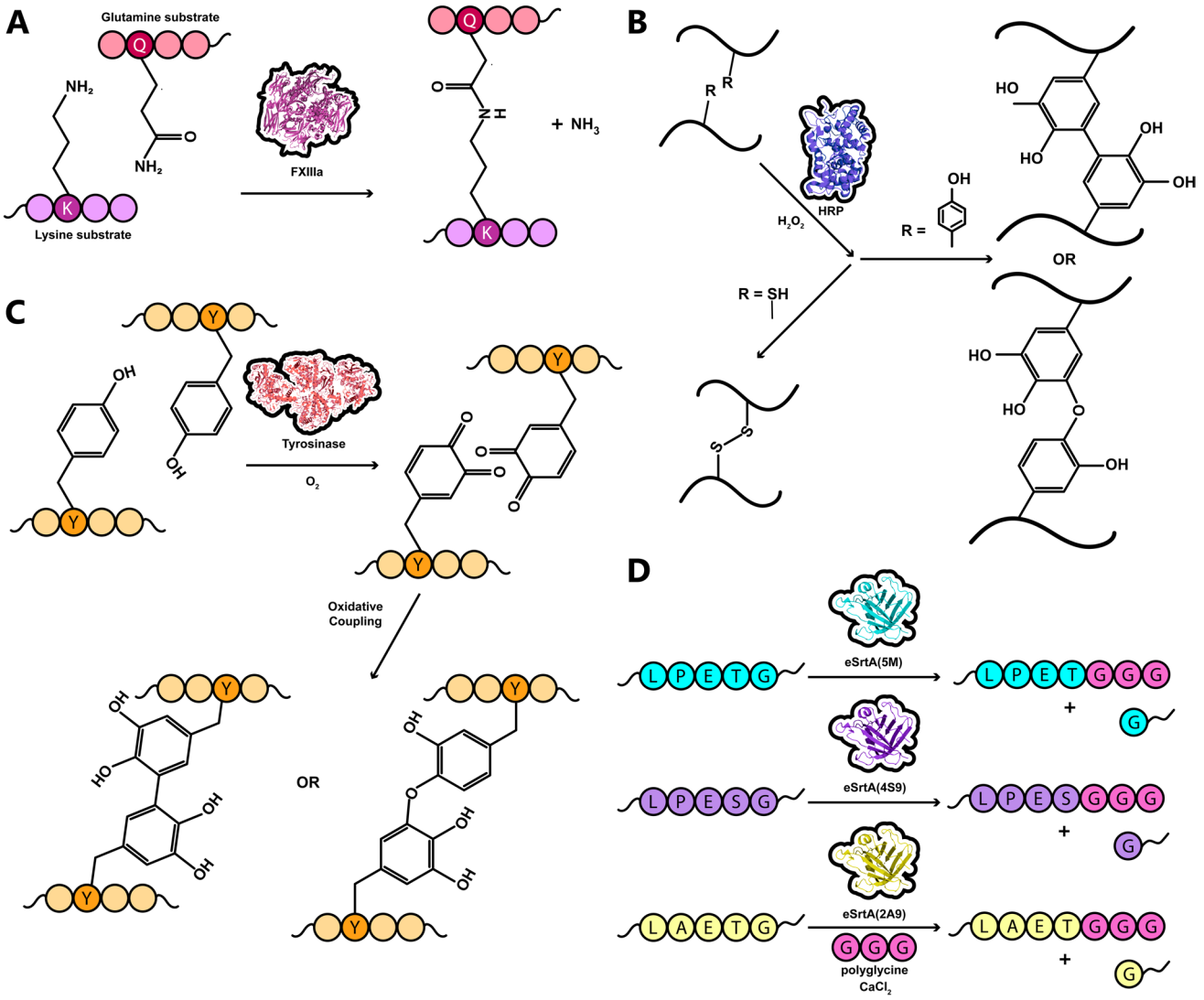


Fig. 1 Major enzymatic reactions employed in hydrogel assembly and disassembly. **A** FXIIIa reaction for crosslinking. **B** Horseradish peroxidase (HRP) oxidation of polymers containing phenols or thiols leads to covalent bond formation. **C** Tyrosinase reaction for oxidative coupling of tyrosine residues. **D** eSrtA(5 M)-, eSrtA(2 A9)-, and eSrtA(4S9)-sortase-mediated crosslinker degradation. PDB: 1f13, 1 gwo, 5 m6b, 1ija

fibrinogen peptides, has been employed for hydrogel degradation and triggered protein release [28–30]. Wiley et al. crosslinked thiol-ene hydrogels with a defined ratio of thrombin-cleavable LVPR↓GS peptides to non-degradable thiolated sequences, permitting dynamic matrix softening and assessment of variable mechanical cues on breast cancer morphology and proliferation; post softening, gels could be orthogonally stiffened by flowing in thiol-ene components [29]. More recently, Zhang et al. crosslinked bilayer hydrogels with a protein-based linker composed of thrombin and hirudin (an inhibitor of thrombin) [30]. Thrombin activity could be reversibly turned on by mechanical stretching, separating thrombin and the inhibitor, and allowing for thrombin to cleave nearby LVPR↓GS crosslinking peptide sequences. If fibrin was flowed into the system, thrombin could induce a strain-stiffening response instead. Sequences sensitive to plasmin or urokinase (upstream of plasmin activation) can similarly be included in PEG or protein-based hydrogels for material degradation [31–33].

One major drawback with these approaches is the difficulty in expressing many of these species recombinantly. To complicate the process even more, most of these enzymes cannot be expressed in their active form and must be first catalytically processed by a different protease, requiring even more difficult recombinant expression [34]. Thus, research groups utilizing FXIIIa and thrombin are typically relegated to purchasing purified proteins or extracting it themselves from donor blood.

Oxidoreductases (Horseradish Peroxidase, Glucose Oxidase, and Tyrosinase)

Horseradish peroxidase (HRP) is a metalloenzyme catalyzing the oxidation of many substrates by hydrogen peroxide and is commonly used in the biosciences in techniques such as western blotting and immunoassays. The resting ferric enzyme first reacts with H_2O_2, generating an active intermediate, which is then reduced to the resting state by reactions with reducing substrate molecules [8]. In the context of biomaterials, it has been primarily exploited to crosslink phenol moieties in tyramine-modified gelatin or silk (Fig. 1B) [8, 35, 36]. The use of H_2O_2 as a starting substrate may be problematic for applications involving cell encapsulation, as high concentrations are cytotoxic; Kim et al. first proposed using HRP in tandem with glucose oxidase (GOx) to generate a gradual source of H_2O_2 as it oxidizes free glucose to form hydrogels [37]. Gantumur et al. reported a related crosslinking mechanism, whereby HRP was used both as a catalyst and supplier of H_2O_2; the authors hypothesized that HRP oxidizes thiols on itself to generate H_2O_2 [38]. HRP has also been shown to mediate thiyl radical formation for thiol-norbornene crosslinking, which can be enhanced by the inclusion of nearby tyrosine residues that can efficiently transfer radicals to the cysteine groups [39]. The ubiquity of this enzyme and its wide palette of substrates make this reaction an attractive choice for biomaterial formation; though in-house recombinant expression again remains difficult.

GOx, a dimeric glycoprotein extracted from several fungi and insects, has been used by itself as well to initiate radical chain polymerization of vinyl monomers. This enzyme depends heavily on a bound cofactor, flavin adenine dinucleotide (FAD), to catalyze the oxidation of β-D-glucose into D-glucono-1,5-lactone. In this redox reaction, FAD is the initial electron acceptor, which is reduced to $FADH_2$; the reduced cofactor is then reoxidized by molecular oxygen (O_2) to yield H_2O_2 (Fig. 2A) [40]. The in situ-generated H_2O_2 can then be converted into initiating radicals through multiple methods: the most explored in the hydrogel field has been through Fenton chemistry, whereby the oxidation of Fe^{2+} to Fe^{3+} concomitantly reduced hydrogen peroxide to hydroxyl radicals, which can then initiate the polymerization reaction (Fig. 2B) [41]. Compared to other radical polymerization strategies, GOx initiation is resistant to oxygen inhibition—a common issue in many radical propagation chemistries.

GOx-mediated acrylate polymerization has been used extensively by the Bowman and Anseth groups to create layered core–shell bulk hydrogels, particles, and thin films [41–45]. The benefit of this approach is the high control over

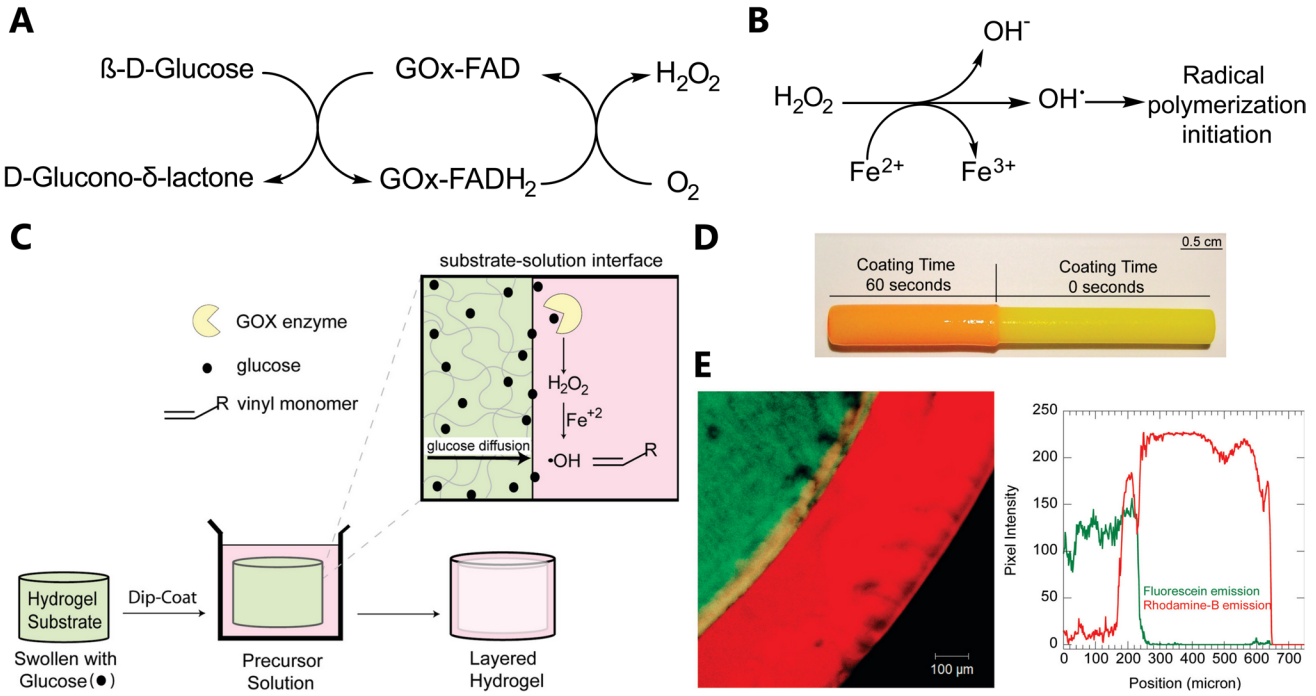


Fig. 2 Glucose oxidase reaction for vinyl polymerization. **A** GOX-FAD catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone. **B** Fenton reaction to generate hydroxyl radicals for initiating radical polymerization. **C** Stepwise process to generate a 3D layered hydrogel. Initially, hydrogel is swollen in glucose solution and then dip-coated in glucose oxidase and vinyl monomer solution, which then generates a layered hydrogel. **D** Image showing increase in layer deposition post 60 s of dip coating. **E** Fluorescent cross-sectional images of a layered hydrogel and quantification of fluorescence radially. Images adapted and reproduced with permission from [42]

interfacial events due to the highly specific binding affinity between β-D-glucose and GOx and the relatively accessible and cheap components (GOx can easily be purchased). Glucose can be preswollen into a bulk hydrogel, spatially confining its location; dipping the hydrogel into an aqueous precursor solution (containing GOx, monomer, and Fe^{2+}) initially results in localization of the enzymatic reaction at the surface (Fig. 2C). As glucose and hydrogen peroxide rapidly diffuse from the hydrogel into the surroundings, the reaction zone is extended, resulting in the formation of relatively thick (150–650 μm) hydrogel interfaces via a frontal polymerization. Manipulating reaction conditions such as immersion time, glucose concentration, or monomer fraction resulted in different layer thicknesses (Fig. 2D, E) [42]. Similarly, initial encapsulation of GOx or the ferrous ions, as opposed to glucose, controlled particle shell thickness [43]. As the gel forms, GOx becomes entrapped in the polymer network; secondary layers can then be formed by leveraging the diffusion of GOx into a second aqueous solution [44]. Intriguingly, this reaction has also been used to study cellulase activity: as cellulose fibers were broken down into glucose in a GOx and monomer precursor solution, a thin fluorescent hydrogel was formed in that location, allowing for visualization [46]. Such an approach could allow for localized biosensing of gluconeogenesis in hepatic tissue constructs [47]. While GOx coupled with Fenton chemistry has been used successfully (and perhaps somewhat surprisingly) to encapsulate cells, the production of H_2O_2 continues to pose an issue for viability; thus, the inclusion of catalase, which reduces H_2O_2 to water and O_2, is recommended [41]. This chemistry is perhaps best suited for creating layer-by-layer structures for controlled release applications as opposed to cellular encapsulation.

Tyrosinase, an oxidase involved in melanin production across many species, carries out the oxidation of phenols, such as those found in tyrosine and dopamine, to quinones in the presence of oxygen. The highly electrophilic quinones can participate in a wide variety of reactions such as Michael or Schiff base addition, and they can also undergo oxidative coupling with other nearby quinones to form dityrosine bonds (Fig. 1C) [8]. As many proteins contain tyrosine residues, little material engineering is required for basic gel formation: this method has been applied to crosslink natural biomaterials such as silk and gelatin [48]. However, tyrosine is not a common amino acid (for instance, making up only 0.2% of collagen I amino acid composition), and without any extra chemical steps to enrich phenol groups, these gels are only sparsely crosslinked and are quite soft [49]. While gel stiffness is, of course, a function of concentration of polymer chains, early efforts crosslinking low weight percent gelatin (2 wt%) and chitosin (0.24 wt%) blends only achieved shear moduli ranging from 1 to 100 Pa, moduli that are significantly softer than most tissues in the body, aside from adipose and brain tissue [50–53]. Thus, others have utilized tyrosinase as a method to selectively stiffen hydrogels previously formed via an orthogonal chemistry; gelatin- and PEG-based hydrogels secondarily crosslinked with tyrosine-containing peptides have been employed in this regard [39, 54–56]. While most of these hydrogel chemistries rely on tyrosine dimerization, Jonker et al. demonstrated that tyrosinase could catalyze the oxidation of 3,4-dihydroxyphenylacetic acid to react with a ring-strained bicyclononyne, in a reaction reminiscent of strain-promoted azide-alkyne cycloaddition (SPAAC); these two reactions could be used in concert due to differences in kinetics [57]. Though most hydrogel applications utilize mushroom tyrosinase from *Agaricus bisporus*, given its robustness in recombinant expression, tyrosinase derived from *Streptomyces avermitilis* was able to induce almost immediate (50 s) polymerization and could be used in a sprayable HA solution [58]. Due to the high reactivity of quinones with many chemical moieties and the comparative ease of expression and extraction, this enzymatic reaction could see an increase in usage for biomaterial formation and modification. Furthermore, the isolation of tyrosinases from different melanin-producing species, those which are functionally similar but structurally distinct, could expand the utility and further improve kinetics of the reaction. Perhaps a major goal for this area of research would be to improve the recombinant expression and solubility/stability of human tyrosinase, so as to avoid potential immunogenic side effects of bacterial- or fungal-derived species [59].

Sortase

The final major class of enzymes utilized to date for hydrogel assembly and decoration are sortases—bacterial cysteine transpeptidases that covalently anchor proteins to the peptidoglycan cell wall of Gram-positive bacteria. The most prevalent sortase (SrtA) in the biosciences is derived from *Staphylococcus aureus* which recognizes the peptide sorting sequence "LPXTG" (where X is any amino acid); sortase cleaves between the threonine and the glycine, generating an active acyl intermediate, which is subsequently resolved by the nucleophilic attack of the N-terminus of a polyglycine substrate (Fig. 1D) [60]. Of note, the sorting sequence is exceptionally rare in mammalian biology—eukaryotic cells do not express the sorting sequence on membrane-bound proteins, allowing for researchers to use SrtA in the presence of and to even tag mammalian cells [61]. Protein engineers have co-opted this bacterial mechanism to staple a range of macromolecules together with only a relatively small scar [62, 63].

Despite its many benefits, the wild-type SrtA suffers from a few drawbacks. Namely, the enzyme (1) is calcium-dependent, which may be detrimental in certain cases and

(2) suffers from low turnover rate, necessitating the use of equimolar amount of substrate and enzyme with long incubation times [61]. Towards the first point, there have been efforts to engineer calcium-independent variants of SrtA: Hirakawa and colleagues substituted two Glu residues in the β6/β7 loop involved in Ca^{2+} binding to Lys/Ala and Lys/Gln; unfortunately, this variant showed reduced catalytic rates compared to the wild-type [64]. With respect to the other two points, the Chen group has employed directed evolution and yeast surface display to enhance the catalytic activity of SrtA and successfully evolved a variant with five mutations (pentamutant, or eSrtA(5M)) that had a 140-fold increase in LPETG-coupling activity as compared to the wild-type [65]. Subsequently, random mutagenesis of eSrtA(5M), led to a heptamutant variant (eSrtA(7M)), which exhibited a fivefold increase in activity [66]. eSrtA(5M) and eSrtA(7M) have replaced the usage of wild-type SrtA and have even been further mutated to be Ca^{2+}-insensitive [61, 67]. Additional directed evolution efforts have yielded variants that recognize distinct substrate motifs with high specificity, such as eSrtA(2A9) and eSrtA(4S9) which respectively cleave LAXTG and LPXSG motifs [68]. Serendipitously, we have found that eSrtA(2A9) is also calcium-insensitive [69].

The Griffith group pioneered the usage of eSrtA(5M) to immobilize bioactive epidermal growth factor (EGF) in PEG hydrogels [70]. The Zenobi-Wong group creatively employed a photocaged polyglycine probe in conjunction with eSrtA(5M) to 3D pattern the immobilization of full-length and bioactive proteins throughout hydrogels [71]. Additionally, our lab has utilized eSrtA(5M) to site-specifically functionalize proteins with bioorthogonal handles enabling their photopatterned immobilization/release and Boolean logic-based delivery from dynamic biomaterials [72–79]. Further exploiting this unique chemistry, other groups soon followed suit in utilizing SrtA to assemble hydrogel networks, which was found to be more efficient than FXIIIa [34, 54, 80]. While still relatively less popular than the previously mentioned reactions, SrtA reactions offer an easy way to tether proteins into gels or building macromolecular structures with only little protein engineering or synthetic chemistry knowledge.

Since SrtA's reaction mechanism catalyzes a bond breakage between the N- and C-terminus of its peptide recognition motif, this enzyme has also found substantial use for degrading hydrogels in a mild, cytocompatible, and largely "biologically invisible" manner. A series of studies from the Griffith group demonstrated the utility of the eSrtA(5 M) variant to rapidly degrade PEG-hydrogels crosslinked with the LPRT↓G peptide-containing motif by inundating the system with free polyglycine, Ca^{2+} (if conducting the reaction not in cell media, which usually contains calcium), and the transpeptidase [81–83]. With these systems, the researchers were able to collect nascent ECM proteins from the PEG-based gels to study the secretome of endometrial cells, as well as recover and subsequently passage large, multicellular gut organoids. A study from the Harley group compared gel degradation by various collagenases and eSrtA(5M) and showed that sortase perturbed murine hematopoietic stem cell surface marker expression significantly less than the other methods [84]. Given the unique reversibility of the SrtA reaction, it has also been used to cyclically modulate hydrogel mechanics: pendant LPRTG and GGG moieties were included PEG arms, which could be joined by sortase treatment to stiffen the hydrogel, the reaction products of which could be subsequently cleaved by addition of sortase and soluble polyglycine [80]. However, the system could only undergo a low number of cycles, as during the acyl-intermediate step, if a water molecule instead of the polyglycine attacks, it yields a dead-end hydrolysis product.

In our group, we have recently explored the usage of three orthogonal SrtA variants for controlled degradation of multimaterial constructs [69, 85]. Bretherton et al. synthesized peptide crosslinkers that were sensitive to eSrtA(5M), eSrtA(2A9), and eSrtA(4S9); treatment with one variant would degrade only the desired region, releasing the cells in that specific hydrogel mixture. To further qualify the "biologically invisible" nature of SrtA, bulk RNAseq was used to assess transcriptomic changes of primary cells in response to treatment with the different variants. As compared to PBS treatment, eSrtA(2A9) and eSrtA(4S9) only exhibited 4–5 differentially expressed genes, whereas treatment with the gold standard, eSrtA(5M) resulted in 89 differentially expressed genes. The three variants proved to be excellent tools for multiplexing stimuli in hydrogels. Our follow-up study further exploited these variants to reversibly stiffen/soften, as well as fully degrade, cell-laden interpenetrating polymer networks; we used these unique materials for studying hMSC cell morphology and differentiation and colon cancer metabolic changes in response to dynamic mechanical cues [85]. While we have conducted transcriptomic analyses, perhaps the next step for further validation of bioorthogonality would be through proteomics, as transcription levels are not always representative of protein expression levels.

In sum, SrtA offers a valuable "biologically invisible" enzymatic signal for (de)decorating and (dis)assembling hydrogel structures. It is readily expressed and purified in high yields from *Escherichia coli* and can be evolved to recognize different sequences with high specificity. Potential drawbacks of using SrtA for hydrogel assembly include the reaction's reversibility and hydrolytic susceptibility, though this is of no concern when utilized for network degradation.

Other Enzymatic Reactions for Supramolecular Assembly

The final, albeit less common, use case for enzymatic reactions for hydrogel synthesis is for endowing

stimuli-responsivity in supramolecular peptide hydrogelators. Typically, these peptides consist of short amino acid sequences, modified with hydrophobic aromatic residues, such as *N*-(fluorenyl-9-methoxycarbonyl) (Fmoc) groups, which self-assemble in water through noncovalent interactions to form 3D supramolecular fibrous structures [86]. To gain temporal control over fiber assembly, numerous groups have explored cleaving hydrophilic groups (e.g., phosphate), with enzymes like alkaline phosphatase (ALP), from the precursors to make hydrogelators (Fig. 3A) [87–92]. Inversely, a kinase can phosphorylate the hydroxyl group, thus reverting the gel to the sol phase, creating a reversible system [93]. Similarly, other enzymes such as MMP-9 [94], β-lactamase [95], and many more described thoroughly elsewhere [86], have been used to cleave off hydrophilic oligopeptides and promote self-assembly.

While cleavage of bonds is the predominant approach, Ulijn and coworkers postulated that reverse hydrolysis of Fmoc amino acids and dipeptides by a protease (e.g., thermolysin) could link these two precursors to form amphiphilic Fmoc-tripeptides [97]. Typically, amide formation is thermodynamically unfavored, but by concentrating the substrates or conducting the reaction in an organic co-solvent, the favored direction of the reaction can be reversed by stabilization of the amide component through self-assembly (Fig. 3B) [98]. A subsequent study explored the potential for a reversible system using thermolysin to assemble the nanofibers and subtilisin, an esterase, to catalyze the disassembly [99]. More recently, Pappas and colleagues created, using this process, a dynamic combinatorial peptide library. A mixture of dipeptides was exposed to thermolysin, which caused a dynamic exchange of peptide sequences; those that resulted in favorable self-assembly are then characterized via

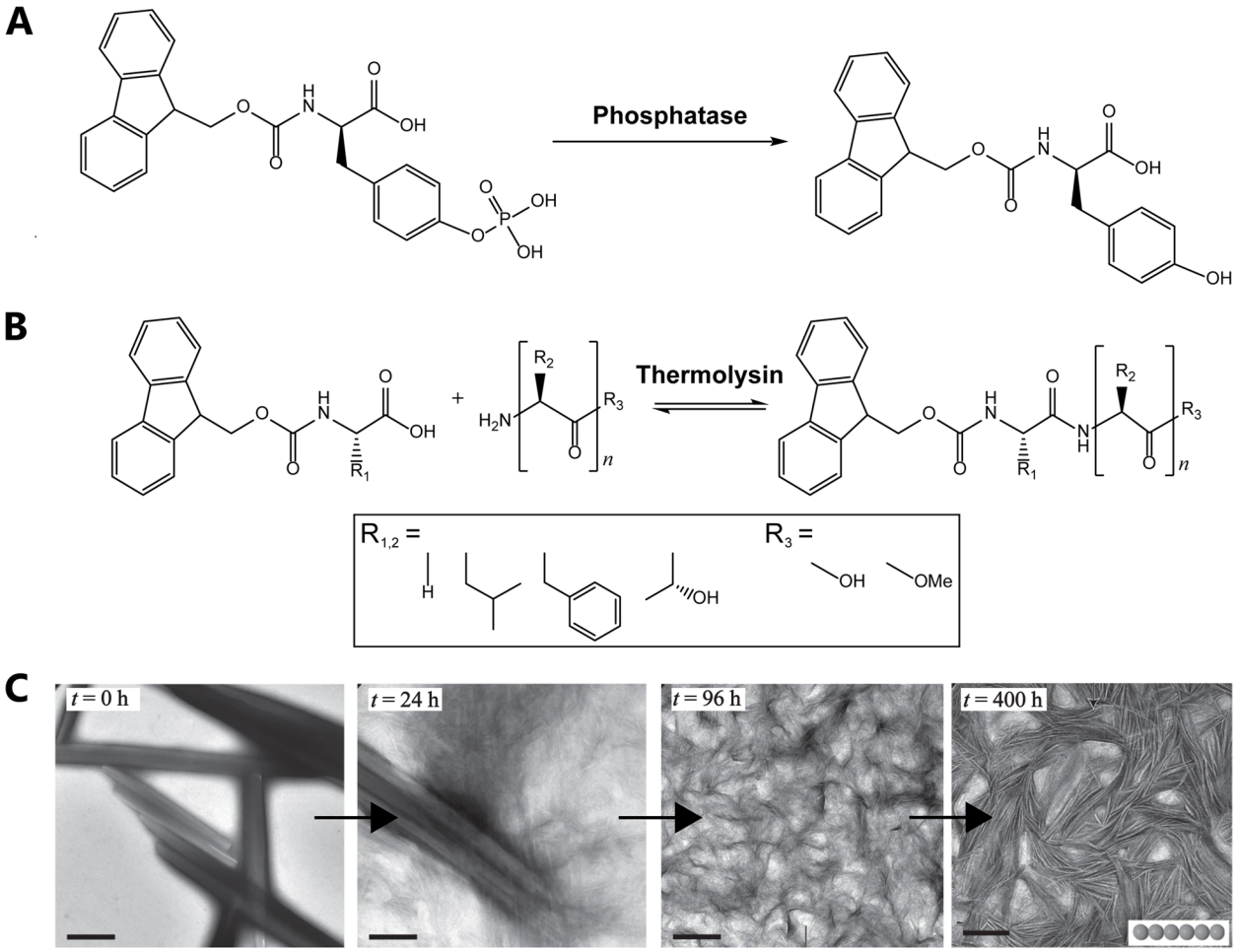


Fig. 3 Enzymatic reactions to mediate self-assembly of peptide hydrogelators. **A** A hydrophilic phosphate group is cleaved off by a phosphatase, resulting in a sol–gel transition. **B** Reversed hydrolysis reaction to produce amphiphilic peptide hydrogelators. **C** Amphiphilic peptide hydrogelators self assemble into fibrous nanostructures upon the addition of thermolysin. Combinatorial peptide libraries in tandem with reversed hydrolysis can dramatically increase the design space of these hydrogelators. Scale bars = 200 nm. Images reproduced with permission from [96]

mass spectrometry and other analytical techniques (Fig. 3C) [96]. These reactions, while ostensibly creating a more complicated system than simply starting with Fmoc-containing oligopeptides, could find a use-case as injectable, self-assembling structures in vivo, by harnessing tissue-specific enzymes.

Conclusions and Future Outlook

Enzymatic reactions are a valuable and versatile instrument in the biomaterial researcher's toolkit. They offer mild and biocompatible reaction conditions, utilize reagents that can be either recombinantly expressed in-house or are commercially available, are straightforward to employ, and require no additional equipment to use. While designing a hydrogel system, certain factors must be considered, such as biocompatibility if the system is to be formed/degraded in the presence of cells and bioorthogonality if the system is for studying matrix stiffening/softening, and cost and accessibility. All of the systems proposed here are cytocompatible, though prolonged treatment with factors such as MMPs, trypsin, or collagenase may impact cellular integrity. Some enzymes benefit from their promiscuity, enabling them to be applied to many biomaterial contexts without additional synthetic load but are likely to promote unwanted reactions that muddle the underlying biological questions being posed. Others, including SrtA, offer a distinct advantage by acting on sequences not commonly found within the mammalian proteome, but require more specialized precursor synthesis. Finally, cost and accessibility must be accounted for, as hydrogel geometry and scale would impact the amount of enzyme needed, and purchasing large enzyme quantities may be less practical than recombinantly expressing them in house. However, recombinant expression does require additional instrumentation and start-up costs.

As we look forward, enzymatic reactions can be used in tandem for bond formation or cleavage with other bioorthogonal chemistries, including those mediated with light, to create more complex systems to mimic dynamic biological processes or to create precise drug release systems [29, 54, 85, 100]. As the field progresses, systems that can fully degrade to release cells for downstream studies with little genomic perturbation will become vital towards furthering our understanding of matricellular interactions. Chemistries such as light-induced dimerization or other bond-formation schemes paired with total gel degradation via SrtA treatment could prove valuable for studying fibrosis and tumor development and resolution with more advanced techniques. Other enzymes, such as tobacco etch virus (TEV) could be employed to further multiplex enzymatic reactions in both synthetic and recombinantly expressed hydrogel platforms, though further exploration of the bioorthogonality of these approaches is warranted [101, 102]. Finally, the space of possible orthogonal enzymatic reactions is ever-expanding with the advent of de novo protein design [103]. In the future, we may see developments in protein design that is far outside of mammalian biology, with even less immunogenicity than SrtA variants and with higher catalytic rates. In closing, the hydrogel field is primed for innovations using enzymatic reactions for hydrogel synthesis and dissolution.

Author Contribution I. K. and C. A. D. wrote the manuscript.

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Declarations

Competing interests The authors declare no competing interests.

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