

Modifying Naturally Occurring, Nonmammalian-Sourced Biopolymers for Biomedical Applications

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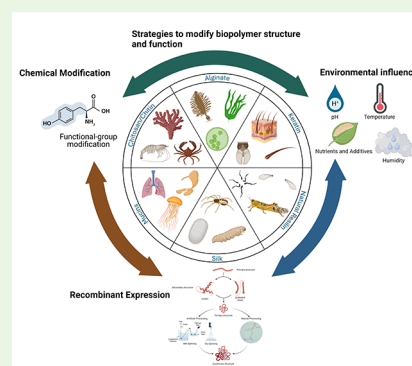
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ABSTRACT: Natural biopolymers have a rich history, with many uses across the fields of healthcare and medicine, including formulations for wound dressings, surgical implants, tissue culture substrates, and drug delivery vehicles. Yet, synthetic-based materials have been more successful in translation due to precise control and regulation achievable during manufacturing. However, there is a renewed interest in natural biopolymers, which offer a diverse landscape of architecture, sustainable sourcing, functional groups, and properties that synthetic counterparts cannot fully replicate as processing and sourcing of these materials has improved. Proteins and polysaccharides derived from various sources (crustaceans, plants, insects, etc.) are highlighted in this review. We discuss the common types of polysaccharide and protein biopolymers used in healthcare and medicine, highlighting methods and strategies to alter structures and intra- and interchain interactions to engineer specific functions, products, or materials. We focus on biopolymers obtained from natural, nonmammalian sources, including silk fibroins, alginates, chitosans, chitins, mucins, keratins, and resilins, while discussing strategies to improve upon their innate properties and sourcing standardization to expand their clinical uses and relevance. Emphasis will be placed on methods that preserve the structural integrity and native biological functions of the biopolymers and their makers. We will conclude by discussing the untapped potential of new technologies to manipulate native biopolymers while controlling their secondary and tertiary structures, offering a perspective on advancing biopolymer utility in novel applications within biomedical engineering, advanced manufacturing, and tissue engineering.

KEYWORDS: biopolymers, natural products, genetic engineering, chemical modification



1. INTRODUCTION

The utilization of natural biopolymers in biomedical applications has a broad and rich history. Evolution has generated expansive natural biodiversity, resulting in a variety of distinct secondary and tertiary structures, diverse functional groups, a wide range properties, and highly variable thermodynamics that can be leveraged to address current and future healthcare challenges. However, despite the potential of utilizing the rich biodiversity of natural biopolymers, translation to the clinic has proven challenging.¹⁰

Ideally, the biomedical field should strive to create solutions that combine the structural complexity of natural biopolymers with the benefits of traditional polymer synthesis, achieving high levels of process standardization that minimize variability over time. Natural biopolymers, such as proteins and polysaccharides, are produced from amino acids and sugars, originating from diverse sources, ranging from human or animal tissues, plant tissues, invertebrates, and insects (Figure 1A). In this review, we aim to highlight the potential of natural biopolymers, while discussing the challenges and limitations for use, highlighting methods for modifications that might be

necessary to enable process standardization, purification, and/or materials fabrication.

The clinical translation of naturally derived materials encounters several hurdles (Figure 1B), including issues in reliable and scalable sourcing of biopolymers that show favorable properties, as well as demanding purification and processing parameters such as solubility constraints¹⁰ and the inability to remove contaminants such as endotoxins. To address these issues, synthetic polymers have been engineered to mimic natural biopolymers while reducing batch-to-batch variability and providing methods to precisely control the monomeric sequence. However, synthetic materials often fail to replicate the full diversity of functional groups, interaction kinetics, and secondary or tertiary structures found in native

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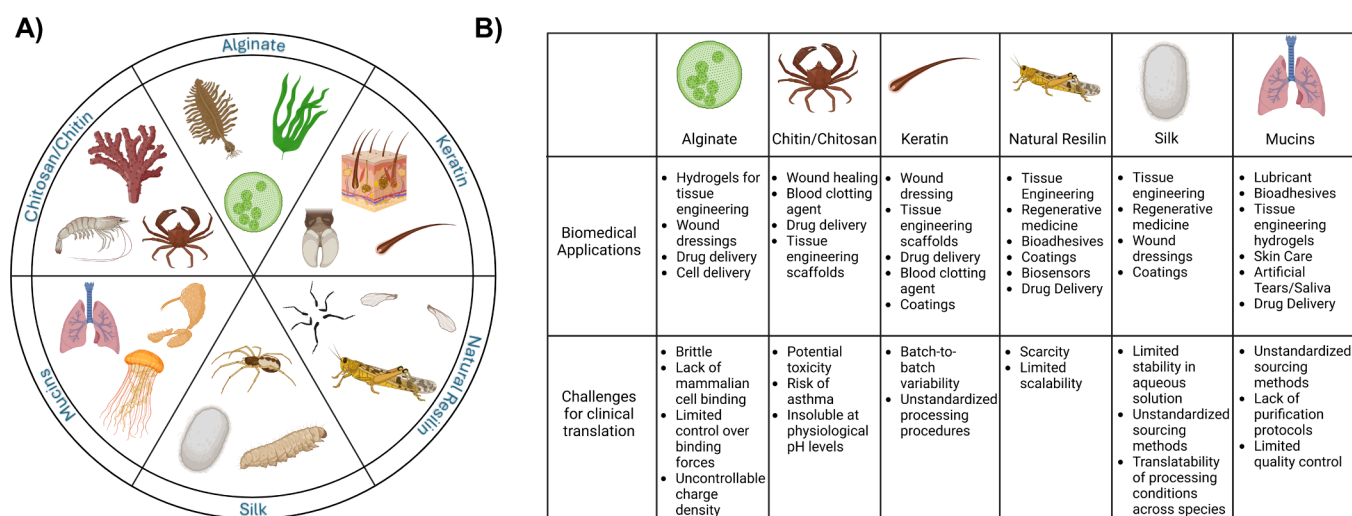


Figure 1. Clinical translation challenges in utilizing the diverse sourcing of common natural biopolymers for biomedical applications for clinical translation. A) Biopolymers originate from a range of sources with the potential to offer unique biopolymer properties to diversify the array of natural materials available for healthcare applications. B) Investigators have harnessed the diverse array of biopolymers to tackle various biomedical needs. However, each biopolymer, and even species-to-species variations, presents unique challenges that impede its broad implementation in clinical settings. Known uses and current challenges for clinical translation for alginate, chitin/chitosan, keratin, resilin, silk, and mucins are provided. Schematics were created with a license from [BioRender.com](#).

biopolymers. To bridge this gap between synthetic systems and naturally sourced materials, short peptides or biopolymer fragments can be produced in nonmammalian systems like *Escherichia coli* (*E. coli*)¹¹ or *Bacillus megaterium* (*B. megaterium*),¹² providing alternative sources for the bioactive components of natural biopolymers. For some systems, such as spider silk¹³ or resilin-like polypeptides,^{14,15} investigators have overcome some production and purification hurdles to create functional materials. However, for other systems, scaling up production within microbial systems can be challenging due to metabolic burden and limitations in collection and purification.^{16–18} Moreover, the organism used for recombinant production significantly influences biopolymer structure and functional properties, with variations in glycosylation and post-translational modifications among plants, insects, mammals, and bacteria.^{19–27}

As methods continue to advance to improve native structures and production techniques, investigators can expand the range of natural materials and properties available for biomedical applications. These applications include clinical products, model systems, and culture platforms. This review highlights commonly used nonmammalian natural biopolymers in biomedical applications, emphasizing both their successes and challenges in laboratory settings and translation to clinical use. Additionally, it provides insights into the strategies employed by investigators including chemical modification, recombinant expression technologies, and environmental regulation to accentuate the native biopolymers and overcome existing limitations, offering a comprehensive perspective on the evolving landscape of biopolymer utilization in biomedicine.

2. NATURALLY DERIVED BIOPOLYMERS IN HEALTHCARE: ADVANTAGES AND CHALLENGES

Naturally derived materials are defined within this review as materials produced by a living organism and then used as functional material, especially focused on biomedical applications. We define naturally derived biopolymers as large

proteins or polysaccharides purified from the original natural sources (plant, insect, amphibian, etc.), that can then be used to reform higher order structures and materials. We focus on biopolymers that can be altered through processing or chemical modification once obtained from their natural state. Furthermore, many biopolymers can be produced by recombinant technologies (e.g., in a microorganism) as an alternative source for the biopolymer of interest; we distinguish between these recombinantly produced biopolymers and the naturally derived biopolymers for the purposes of this review, as characteristics such as glycosylation patterns, post-translational modifications, and secondary structures may differ as a function of biopolymer source and purification strategy.

Proteins are diverse biopolymers composed of amino acids that can take on highly specific structures and functions such as specific integrin binding motifs such as Arg-Gly-Asp (RGD).²⁸ This specificity can be leveraged to tackle numerous healthcare applications where specific interactions with cells and tissues is important. Additionally, various proteins, while not necessarily providing significant biological activity, can offer a diverse range of mechanical properties, which will be highlighted throughout this review. However, the advantages due to the high specificity of structure can often be compromised due to proteins' limited stability under a range of environmental conditions, which can alter the structure that provides the desired functionality. Conversely, polysaccharides, composed of monosaccharides linked by glycosidic bonds, typically have simpler structures compared to proteins, but offer greater stability of structure and properties over a wider range of environmental conditions. Additionally, this simple carbohydrate-based structure often elicits a lower immunogenic response compared to proteins, making polysaccharides excellent candidates for bioadhesives and drug delivery systems (Figure 1B).

A synthetic version of a naturally derived biopolymer would be one produced or modified through synthetic chemistry or traditional polymer chemistry. Engineered naturally derived biopolymers refer to biomaterials produced within a model

organism that differs from its natural host. In this process, genetic engineering strategies are employed to transform or transfect an organism, enabling the production of biomimetic biopolymers. Similarly, an engineered peptide represents the production of a shorter chain of a protein-based biopolymer in its non-native host. Briefly, we outline the advantages and challenges for using alginates, chitosans, keratins, mucins, resilins, and silk fibroins (Figure 1B).

2.1. Sourcing Natural Biopolymers. Some classes of biopolymers are made by many organisms, but currently, the biomaterials community has routinely focused on only one or two species for most biomaterials' development. For example, for silk fibroin-based biomaterials, it has been reported that roughly 90% of silk-based manuscripts in tissue engineering up to 2019 were from the domesticated silkworm, *Bombyx mori* (*B. mori*).²⁹ Alternatively, in some cases the community has done a great job in leveraging biodiversity. Alginate is a gold-standard example for how naturally derived biopolymer sourcing can yield a range of products. For example, alginate polysaccharides can be harvested from a few species of sea kelps (also known as brown algae (*Phaeophyceae*)), including *Ascophyllum*, *Durvillaea*, *Ecklonia*, *Laminaria*, *Lessonia*, *Macrocystis* and *Sargassum*. Commercially available alginates are primarily extracted from *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria japonica*, *Ascophyllum nodosum*, and *Macrocystis pyrifera*.^{30,31} These species, native to different hemispheres and climates, have the same basic structure, but are not structurally or compositionally identical.³² Moreover, overall yield of alginate biopolymer from a given mass of sea kelp often varies between species, complicating production pathways.

NovaMatrix purifies alginates of different compositions for their product line PRONOVA UltraPure or Sterile Sodium Alginates, which are available at 3 viscosity and molecular weight combinations and at two different monomer unit ratios. The wide range of alginate products available represents the variability in the alginates produced by brown seaweeds and years of efforts, before commercialization, to develop consistent and reliable separation and purification protocols and understand their impact on cell-material interactions.^{33–39} Similarly, hyaluronic acid, a mammalian polysaccharide known as a glycosaminoglycan, was initially sourced from rooster combs or the bovine vitreous humor prior to the development of current production strategies that leverage bacterial fermentation of mammalian polysaccharide sequences, usually strains of *Streptococcus* or *Bacillus*.^{40–43} Like alginates, one can purchase hyaluronic acids with differing intrinsic viscosities, which suggests differences in biopolymer molecular weight, but one can also choose different biological sources for the hyaluronic acid biopolymer. While hyaluronic acid-based biomaterials are outside the scope of this review as they are a mammalian protein, they have been investigated extensively in both their unmodified and modified forms.^{44–46}

These recognitions by the biomaterial industry highlight the known value in using different sources of a given biopolymer for different clinical and preclinical goals. To us as authors of this review, we find that this highlights the benefits of leveraging biodiversity in biomaterials sourcing to identify and generate new biopolymers from similar classes that may have additional benefits to those currently in use, providing new insights for polymer scientists into useful chemistries and fundamental information on what gives each class of

biopolymers their highly sought after and leverageable properties (Figure 1A).

Additionally, when sourcing biopolymers from nonmammalian sources such as insects and crustaceans, ethical considerations are paramount. These organisms must be sourced following sustainable and ecologically friendly methods to ensure humane treatment and the recovery of high-quality and consistent biopolymers. Furthermore, it is crucial to maintain conditions that minimize waste and contaminants through development of environmentally friendly farming and rearing practices. For example, insects are a potential low-cost and renewable food source and several publications have reviewed the ethics of large-scale insect rearing,^{47–49} providing valuable insights for the biomaterials' community. Furthermore, genetic modifications to native insects, plants, or other organisms may pose risks to the natural habitats and ecosystems. Production of genetically modified biopolymers should be a tightly regulated industry, ensuring these organisms are not released into the environment, following strict guidelines and recommendations^{48,50,51} made from other fields.

2.2. Silk Fibers and Silk Fibroins. Silk fibers are a diverse set of proteins spun various arthropod species, including insects and spiders, and are utilized by these organisms as a high-performance material, with a wide range of amino acid sequences and limited sequence homology. In a biomedical context, the predominant naturally derived biopolymers extracted for use are silk fibroins, which are purified from silk fibers produced by insects in the Lepidopteran order (e.g., *B. mori*). Additionally, spidroin proteins, spun by various spiders, have been investigated for a wide range of applications. Sourcing silk fibers directly from spiders poses challenges, necessitating recombinant production of spidroin-like peptides followed by artificial fiber spinning.^{13,52,53} Conversely, silkworms are much easier to cultivate, as demonstrated by the textile industry,⁵² but are much more challenging to produce recombinantly.^{16,17}

Silk fibers derived from silkworms are formed from the self-assembly of many proteins within the silkworm silk gland. These fibers consist of a core of silk fibroin proteins and an outer coating made from a variety of proteins, including sericins, mucins, and seroins. In scientific research, the prevailing silk fibroin source is derived from *B. mori*, the domesticated silkworm, in addition to other high-producing sources including *Antheraea pernyi*, *Samia cynthia ricini*, *Araneus diadematus* and *Trinephila clavipes*.⁵⁴ Different insects naturally produce distinct silk fiber structures, which are diverse in part, due to the differences in the native sequences of the silk fibroin proteins, which make up the majority of the structural components of the fibers, presumed to be driven by evolutionary and environmental pressures.⁵⁵ The fibroin protein itself from *B. mori* is comprised of two main chains, the heavy fibroin chain (molecular weight ~390 kDa) and the light fibroin chain (molecular weight ~26 kDa), and, in silk fiber producing lepidopterans, is accompanied by sericins, a class of molecules acting as a gumming agent around the inner fibroin core of the fiber, when natively produced.^{56,57} In *B. mori*, the heavy chain of silk fibroin contains hydrophobic domains that form a crystalline network when driven to form beta sheets by hydrogen bonding, that subsequently aggregate by removing water from the backbone,^{58–63} rendering *B. mori* silk fibroin proteins water insoluble and facilitating their use as a robust biopolymer that can form a variety of tunable

structures.^{29,56,64} Specifically, *B. mori* silk fibroin proteins can be isolated and regenerated by processing in aqueous media,⁶⁴ an advantage over other biopolymers that require processing in acidic conditions or with organic solvents, which raises questions of safety in biomaterial applications.^{29,65,66} Silk fibroins have been used for a variety of biomedical applications,^{56,57,67–69} including implantable materials for tissue engineering,^{68,70–72} to sensors and soft robotics.⁵⁶ Investigators continue to work toward improvements to overcome challenges in using *B. mori* silk fibroin-based biomaterials, including the lack of protein sequences for interaction with mammalian cells (e.g., integrin binding sites), limited stability of silk fibroin in aqueous solution, and the scale-up of manufacturing and purification processes (Figure 1B).^{54,68} These challenges have provoked interest in the formation of silk fibroin composite materials, chemical modification of regenerated *B. mori* silk fibroin proteins, the production of recombinant silk peptides in microorganisms,^{16,17,73,74} the use of genetic engineering to alter the native protein.^{75,76}

Spider silk fibers, like those derived from silkworms, are also made up of many proteins during the fiber self-assembly process within the spider silk glands. Compared to insects which are only known to produce one heavy fibroin protein, spiders actually produce many spidroin proteins (>15), which can also vary in sequence as a function of habitat.^{77–79} Since the mid-1990s,^{80–82} investigators have been working to improve the expression, purification, and reconstitution of the two main proteins investigated for recombinant expression, major ampullate silk protein-1 (MaSp1) and MaSp2,⁸³ which are responsible for the robust mechanical properties of spider silk fibers. Unlike silk fibers collected from the cocoons of *B. mori*, spider silk fibers must be collected by “milking” a spider to forcibly spin and collect silk fibers.⁸⁴ While this method works for the further assessment of mechanical properties and determination of other chemical, mechanical, and structural characteristics,⁸⁵ it is not a feasible method for the production and scale-up of commercial silk materials. Thus, the majority of efforts toward the development of spider silks for biomedical or commercial applications is in the form of engineered spider silk peptides and methods for expression, purification, and self-assembly of these recombinantly expressed spider silk peptides.^{13,86–88} Alternative production routes, such as the use of plants,⁸⁹ has also been investigated. To get the best of both organisms, investigators have used genetic engineering strategies to produce spider silk proteins in silkworms,^{90–92} a promising new strategy for rational design and production of large silk-like biopolymers.

2.3. Chitosan and Chitin. Chitin and chitosan are aminopolysaccharide biopolymers found in a variety of organisms including insects, crustaceans, mollusks, corals, sponges, and fungi.⁹³ The degree of acetylation is used to distinguish between chitin and chitosan, where chitin is usually described as around a 90% acetylated biopolymer, while chitosan is typically 65% or more deacetylated biopolymer.^{94,95} Chitin is the second most prevalent biopolymer found in nature, leading to approximately one million tons of raw material available for processing per year.⁹⁶ The linear constituent of chitin entails β -(1,4)-N-acetyl-D-glucosamines linked through glycosidic bonds and is naturally found in three isoforms with α - and β - being predominantly used in the formulation of biomaterials.^{95,97–103} Chitin is often leveraged in biomedical applications^{94,96,99–101} due to its thermostability,

chemical tolerance, and natural abundance. However, healthcare applications of chitin face certain limitations, primarily concerning potential toxicity, particularly within the respiratory system (Figure 1B). Previous research has indicated a positive correlation between chitin exposure in the respiratory tracts of mice and asthma.¹⁰⁴ Furthermore, challenges persist in effectively solubilizing and reconstituting chitin and chitosan in aqueous solutions, particularly at pH values relevant to clinical settings.^{94,96,100,101}

Chitosan, which is typically obtained through chemical or enzymatic deacetylation of chitin, has been investigated for a wide range of applications including wound healing and wound dressing, drug delivery and pharmaceutical formulations, and in many micro- and nanoparticle applications.^{94,100,101} Chitosan can also be used to form porous scaffolds via a variety of techniques including electrospinning and gas foaming, and the formation of composite scaffolds with bioactive biopolymers has been studied extensively.⁹⁶ Additionally, chitosan oligosaccharides display minimal toxicity, making them approved as food additives by the American Food and Drug Administration (FDA).¹⁰⁵

2.4. Keratin. Keratins are a class of biopolymers found in soft tissues such as epithelial tissues (“soft” keratins) or found in external, hard tissues such as hooves, hair, and nails (“hard” keratins). Traditionally, hard keratins have been explored for their utility in biomaterials in either a reduced (keratose^{106,107}) or oxidized state (kerateine¹⁰⁸), generated by manipulation of the disulfide bonds present within the biopolymer. Similar to other biopolymers discussed within this review, the extraction and purification of these materials can be challenging or lead to batch-to-batch variability (Figure 1B),¹⁰⁷ but the resulting solubilized biopolymers^{108–112} can be used to form hydrogels, films, coatings, fibers, injectable materials, and porous scaffolds.^{113–120} To overcome some of these sourcing and solubility challenges, methods for optimizing extraction and modification are under investigation.¹²¹

2.5. Alginate. Alginate is a natural polysaccharide-based biopolymer that forms a linear chain of two units: (1–4)-linked β -D-mannuronate (denoted as “M”) residues and α -L-guluronate (denoted as “G”) residues. The ratio of M and G residues dictates the polymer structure and influences maximum ionic cross-linking densities and overall gel state properties. Alginates are water-soluble, forming hydrogels via ionic cross-linking of the G units in the presence of divalent cations, commonly calcium, though barium ions are also suitable. Alginates are commonly harvested from sea kelp or brown algae, and several bacteria also produce alginates. Similar to other naturally occurring biopolymers, the composition and ratio of the two blocks varies between species and the life stage of the species,^{30,122} enabling wide variability in the structure and mechanical properties of the resulting alginate-based biomaterials formed from different alginate sources.^{30,123,124} The biodiversity of the alginates produced affords great flexibility in commercial processing and use, and thus, alginates have been used as a food additive, in wound dressings and pharmaceutical formulations, cell encapsulation, and bioactive molecule encapsulation (Figure 1B).^{10,30,37,125–132}

Encapsulation of cells in alginate hydrogels offers protection from the immune system, affording great clinical promise for cell delivery to patients with autoimmune diseases such as type 1 diabetes.^{133–135} Several limitations surrounding the utilization of alginate biopolymers for healthcare applications

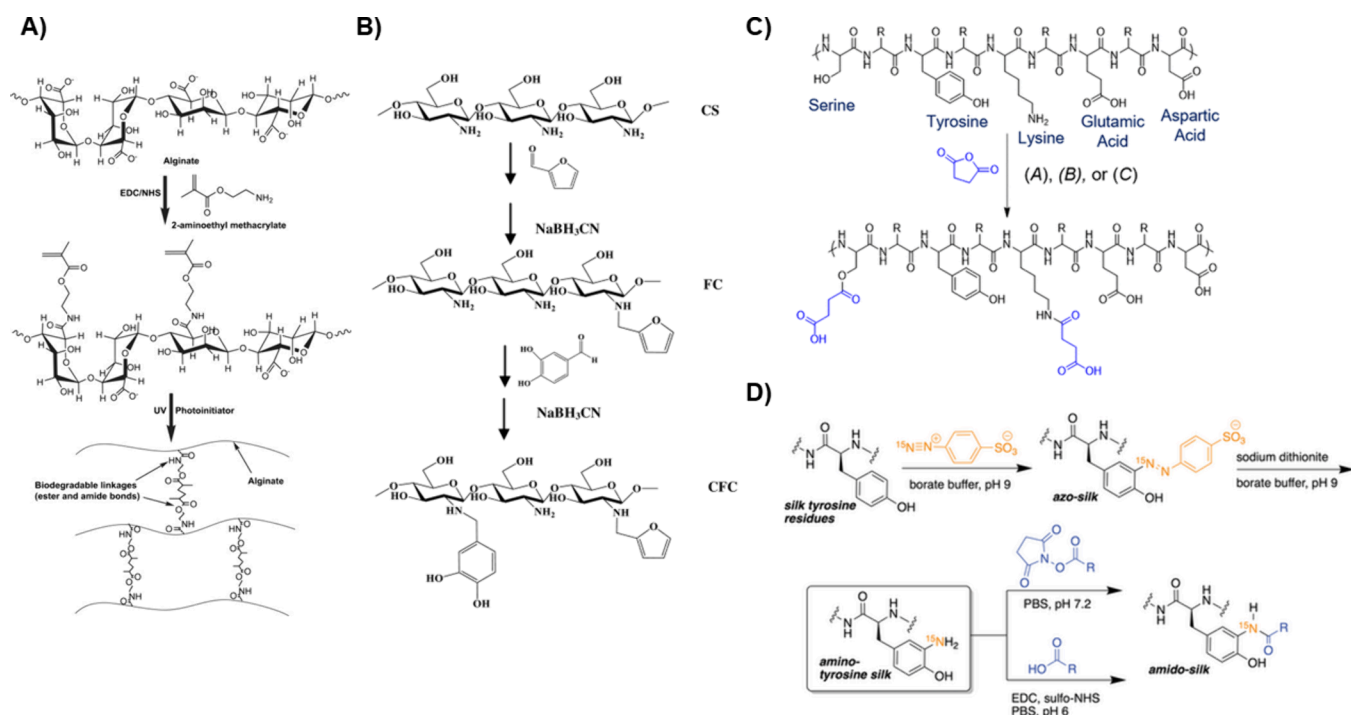


Figure 2. Successful chemical modification strategies for overcoming limitations with natural biopolymers. A) Synthesis of methacrylated alginate through EDC coupling to form photo-cross-linked alginate hydrogels.¹ B) Diels–Alder click reaction for the formation of self-healing chitosan hydrogels.² C) Increasing efficiency of carboxylation of silk fibroin through disruption of noncovalent interactions under mild conditions.⁵ D) Functionalization of tyrosine residues in silk fibroin through an azo-reduction reaction to increase the amount of residues for chemical modification.⁶ (A) was reproduced with permission from reference #1, ©2009 Elsevier Ltd. (B) was reproduced with permission from reference #2, ©2017 Elsevier B.V. (C) was reproduced with permission from reference #5, ©2020 American Chemical Society. (D) was reproduced with permission from reference #6, ©2022 Wiley-VCH GmbH.

include brittle mechanical properties, lack of mammalian cell-binding domains that necessitate the addition of specific integrin binding sites, limited control over the binding forces between the biopolymer subunits during ionic cross-linking, and uncontrollable charge density (Figure 1B).^{30,34,122,136–138} Similar to many other natural biopolymers, investigators aim to overcome these challenges where improvements have been made through modification strategies, such as cross-linking cell-binding epitopes to the material to improve its biological activity.^{30,138} Additionally, improvements have been made in alginate sourcing and batch-to-batch variabilities through the generation of composite materials and interpenetrating networks, modification of the alginate biopolymer backbone to engender a specific biological function,^{30,137,139–141} and strict sourcing, purification processes, and batch-to-batch characterization for commercially available products (e.g., NovaMatrix).

2.6. Resilin. Resilin was first reported and described by Weis-Fogh in the early 1960s and has since been widely found in multiple arthropod species including insects, crustaceans, scorpions, and centipedes.^{142,143} Resilin has captured significant interest in the biomaterials community owing to its remarkable properties, encompassing exceptional elasticity, high extensibility, reversible deformation without loss of energy, and outstanding resilience when subjected to stretching.^{144,145} Furthermore, it is also extremely heat stable, remaining unaltered in neutral water that has been heated to 125 °C and does not degrade until 140–150 °C.^{144,146} Studies with engineered resilin peptides indicate biocompatibility and a lack of unwanted immune responses.^{147,148} Engineered resilin peptides are the predominant material used for regenerative medicine applications as the practical utilization of the resilin

sourced from its natural state remains unfeasible due to scarcity and limited scalability. Over the past decade, the development of resilin biomimetic peptides called resilin-like polypeptides (RLPs),¹⁴⁴ has dominated the discussion for resilin-based biomaterial systems.^{15,144,147–150} Through genetic engineering and recombinant expression, this versatile protein can be manipulated in a variety of ways, allowing RLPs and resulting bioelastomers to have great potential for an assortment of biomedical applications including biosensors, tissue engineering, microfluidic devices, and controlled drug delivery systems (Figure 1B).^{144,151–154}

2.7. Mucins. Mucins are large glycoprotein biopolymers secreted by goblet cells in the epithelium and form many lubricating, hydrogel-like materials within the body, including in the lungs, intestines, and nasal passages, making them a useful and instructive biopolymer for biomaterials development.^{155,156} Mucins contain terminal acidic sugars that impart an overall negative charge in addition to an extended central protein and regions of O-glycosylation, which can be acidic or neutral depending on their origin. Mucins are exploited for their barrier, lubrication, hydration, and bioactivity capabilities, and are found in multiple locations in almost all animals to help protect the epithelium from dehydration, mechanical stress, bacteria, and viruses.^{157,158} Mucins are investigated for their use in skin care products and cosmetics,¹⁵⁹ as artificial tears or saliva,¹⁵⁵ as drug delivery vehicles,^{160–162} and have been shown to have unique antibacterial and immune responses (Figure 1B).^{163,164}

Natural mucins are frequently harvested from porcine and bovine gastric or submaxillary systems,¹⁶⁵ but the use of less common sources of mucins, such as mollusks, could have

potential for large scale production and aid in healthcare materials development.^{166–168} Other novel sources of mucins include jellyfish, which have been used to inhibit degeneration of cartilage in a rabbit model,¹⁶⁹ and snail and slug mucus, utilized for antimicrobial properties^{170–172} and more recently to study wound healing and skin rejuvenation^{173,174} for both cosmetic and medical applications. As with most naturally sourced biomaterials with numerous potential sources, batch-to-batch variability, lack of standardization of purification protocols, and limited quality control practices to maintain purity have limited the commercialization of a naturally derived mucin-based material (Figure 1B).¹⁵⁵ Similar to approaches taken to create resilin-like polypeptides, investigators have leveraged the unique chemical structure and function of mucins toward the development of engineered artificial mucin-like polymers as a way of avoiding issues arising from the lack of standardization in the use of animal-derived mucins.¹⁵⁶ Recombinant expression systems have led to improved batch-to-batch reproducibility and a reduction in contamination risks while continuing to exploit the use of the glycocalyx and complexity of the mucin biopolymer and offer advanced manufacturing potential from a nonmammalian source.^{156,160,175–177}

3. STRATEGIES TO IMPROVE THE UTILITY OF NATURALLY DERIVED BIOPOLYMERS

Nature has evolved a rich landscape of biopolymers that can be leveraged in the medical community to address numerous healthcare challenges prevalent currently and in the future. However, substantial challenges reside in utilizing a vast majority of naturally derived biomaterials including sourcing, batch-to-batch variability due to biological diversity or environmental factors, and constrained capacity to adjust or modify the material properties. Hence, as engineers, we possess the capability to utilize our existing toolsets in order to surpass certain constraints inherent in natural biopolymers that inspire us, paving the way for a fresh wave of innovative biomaterials. Relevant to this review, chemical modification strategies, genetic engineering, and environmental influence will be discussed.

3.1. Chemical Modification. The most common way to enhance the functionality of a naturally sourced biopolymer is to perform a chemical modification at specific amino acid residues or specific side chains to alter the native structure, such as the schematics shown in Figure 2. These chemical modification strategies aim to alter the biopolymer without hurting or diminishing the native features and the ability of these biopolymers to self-assemble. A wide range of chemical modification strategies exists for synthetic polymers and investigators have worked to apply these modification strategies to naturally sourced materials, including silk fibroins,^{178,179} alginates,^{180,181} and keratins.¹⁸² Often, the goal is to introduce reactive side chains to improve the tethering of additional bioactive molecules or alter the ability of the biopolymers to cross-link and form reliable and reproducible networks. Two of the main challenges investigators work to overcome in the chemical modification of naturally sourced biopolymers are 1) limiting the degradation of the protein when using harsh conditions during modification and 2) the extent of modification and recovery of modified biopolymers.

3.1.1. Methods to Methacrylate Natural Biopolymers. Modifying a biopolymer with methacrylate groups enables free

radical cross-linking and is desirable for coupling or gelation reactions using UV light. Many sources use methacrylic anhydride to modify primary amine groups for this purpose, and this approach has been demonstrated on silk fibroin¹⁸³ and chitosan.^{184,185} Another approach is to react methacrylic anhydride with hydroxyl groups on alginate.¹⁸⁶ Other methods to obtain methacrylate functionality have been demonstrated on silk fibroin, which used glycidyl methacrylate,^{187,188} and alginate, which attached 2-aminoethyl methacrylate using EDC coupling (Figure 2A).¹ Covalent cross-linking through the methacrylate groups is particularly useful for additive manufacturing applications, however, a potential downside for the methacrylate reactions is that the method used to cross-link the modified biopolymers may render the material unable to undergo biodegradation.

3.1.2. Carbodiimide Coupling to Natural Biopolymers. 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) is often selected for biopolymer modification because it can be used in aqueous conditions. EDC activates carboxylic acid groups to form an *O*-acylisourea intermediate under mild to slightly acidic conditions, and then primary amine-containing molecules may react with the activated site to result in an amide bond that covalently links the two molecules. The *O*-acylisourea intermediate is susceptible to hydrolysis, resulting in the recovery of the carboxylic acid functional group, so *N*-hydroxysuccinimide (NHS) or *N*-hydroxysulfosuccinimide (sulfo-NHS) is often used in conjunction with EDC to produce an NHS ester intermediate which is more stable, less susceptible toward hydrolysis, and favors the reaction between the carboxyl and primary amine groups. EDC coupling involving alginate and silk fibroin is highlighted below, but it has also been used to modify other natural proteins such as keratin^{189–191} and chitosan.^{192,193}

3.1.2.1. Carbodiimide Coupling with Alginate. Alginate has attracted attention for its rapid and reversible solution-to-gel transitions. Alginate contains no cell-binding sites; however, many pendant hydroxyl and carboxylic acid groups exist and provide opportunities for synthetic modification (Figure 2A). The carboxylic acid groups can be modified with arginine-glycine-aspartate (RGD)-containing ligands using EDC coupling to facilitate cell attachment and enhance interaction between the alginate construct and attached cells, as demonstrated for skeletal myoblasts.³³ Additionally, functionalizing alginate with vinyl ether¹⁹⁴ or tyramine functional groups¹⁹⁵ by EDC coupling produces a dual cross-linked hydrogel, where one set of cross-links forms a physical network (e.g., by hydrogen bonding or ionic cross-linking) and the other forms a covalent network upon exposure to UV light. Since many applications of alginate-based materials exploit the reversibility of the alginate network, care must be taken to ensure that the modifications do not disturb the ability of alginate to be ionically cross-linked.

3.1.2.2. Carbodiimide Coupling with Silk Fibroin. Silk fibroin has been coupled with polysaccharides,^{196–199} proteins,^{200–206} and peptides^{202,205} by activation of native carboxylic acid residues using EDC. A significant challenge is attaining high levels of functionalization, which exists due to low numbers of carboxylic acid reactive sites as well as difficulty reacting to those sites. Methods²⁰³ to increase carboxylic acid content have been reported, however some approaches result in low functionalization yields and significant degradation of the protein.^{207,208} A milder carboxylation route using succinic anhydride in an ionic liquid/dimethylformamide

enabled up to a 90% degree of substitution and showed less protein degradation (Figure 2C).⁵ Carboxylated silk can then be modified using EDC coupling with and without the presence of surfactant. In the presence of surfactants, the more highly carboxylated silk fibroin was more amenable to the EDC coupling reaction, resulting in higher degrees of substitution with greater preservation of the initial silk fibroin biopolymer structure.⁵ Additionally, the generation of amino-tyrosine silks via the formation of azo-silk can lead to the enhancement of tyrosine residues on the silk backbone, providing additional sites for the use of EDC coupling (Figure 2D).⁶ Further work will continue to evaluate how the chemical composition of substitutions affects the self-assembly of the protein and its properties as a biomaterial in addition to considering if these chemistries are effective in modification of nonmulberry silk fibroin sources.

3.1.3. Modification of Biopolymers by Oxidation Reactions.

3.1.3.1. Generation of Carboxylic Acids. Biopolymers featuring carboxylic acid functional groups are a desirable precursor for modification due to the availability of aqueous phase reactions that target this functional group, including the EDC coupling reactions discussed above. To enrich carboxylic acid groups of silk fibroin, sodium hypochlorite has been used to oxidize the hydroxyl groups on serine residues.²⁰⁹ Modified silk had an observed 47% degree of carboxylation and a high reaction yield, though it was noted that too much sodium hypochlorite resulted in significant protein degradation. This carboxylated silk has been blended with chitosan to form composite materials with high moduli via layer-by-layer assembly.²¹⁰ Alternatives to oxidation to generate carboxylic acids include the modification of alcohol groups with an organohalide (e.g., chloroacetic acid²⁰⁸ or iodoacetic acid²¹¹) under basic conditions, which has been shown to modify silk fibroin.²¹² Under some conditions, these methods can prove challenging as the pH adjustments required can result in an impactful decrease in biopolymer molecular weight. Another alternative to oxidation in silk fibroin used an anhydride (e.g., succinic anhydride²⁰⁷) in organic and ionic liquid solvents to modify alcohol and amine groups, which was found to result in high degrees of functionalization and less protein hydrolysis, as indicated by protein molecular weights in excess of 100 kDa.²⁰⁷ More recently, this approach has been confirmed with a different solvent system (1.0 M lithium chloride in dimethyl sulfoxide) that also demonstrated the preservation of carboxylated silk protein with high molecular weight.²¹³ With different options available for carboxylation that require more or less stringent conditions, the user may select the reaction route based on the desired functionalization and molecular weight of the modified biopolymer and subsequent downstream applications.

3.1.3.2. Generation of Aldehydes. Aldehyde functional groups generated on polysaccharides and proteins are not naturally occurring and can be leveraged to permit functionalization at specific sites. Aldehydes may react with primary amine, hydrazide, or aminoxy groups to generate stable linkages for cross-linking or other coupling reactions. For proteins, approaches to introduce aldehydes include enzymatic treatments and N-terminal serine oxidation. Vicinal diols of polysaccharides, including chitosan,²¹⁰ N,N,N-trimethylchitosan,²¹⁴ and alginate,²¹⁵ can be oxidized by periodate (IO_4^-) to generate dialdehydes. Alternatively, primary alcohols can be oxidized to aldehydes using 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO),²¹⁶ which has

been explored in combination with sodium hypochlorite and sodium bromide to oxidize chitosan,²¹⁶ with more than 95% of primary alcohols successfully oxidized.

3.1.4. Less Common Chemical Modification Strategies.

3.1.4.1. Noncovalent Interactions. Host–Guest. Host–guest interactions have been used with chitosan^{217,218} to generate supramolecular gels. One example is materials based on the interaction of β -cyclodextrin (CD), a cyclic oligosaccharide that contains a hydrophobic cavity, with adamantane, which has a high affinity for CD. Chitosan modified with β -cyclodextrin (CD)²¹⁹ was studied for its gelation behavior using adamantane-modified chitosan and adamantane-modified poly(ethylene) glycol (PEG).^{217,4} When CD-modified chitosan was mixed with the PEG-diadamantane guests, the viscosity increased, but a stable gel was not formed. In contrast, when the CD-chitosan was mixed with the adamantane-modified chitosan, a gel-like behavior was observed but the storage and loss moduli were not independent of the frequency, which was attributed to the network reversibly breaking under stress and then reforming. The ability for biopolymers to undergo reversible sol–gel transitions may be adapted for additive manufacturing or may find application as injectable materials.

Boronic Acids. Boronic acids reversibly interact with diols to form boronate esters, making this moiety a promising feature for responsive hydrogels.²²⁰ Boronic acid groups can be attached to alginates using EDC coupling to generate a pH-dependent material. In basic conditions, gels are formed due to boronic acid groups interacting with the neighboring hydroxyl groups, and the resulting gels are self-healing, injectable through a 21G needle, and support the 3D encapsulation of HeLa cells.²²¹ In acidic conditions or when exposed to fructose, the boronic acid-diol network dissociates. In another study of boronic acid-modified alginate, the materials formed hydrogels in phosphate buffered saline (PBS), and the gels demonstrated self-healing and shear thinning ability, as well as strong adhesive properties and low toxicity *in vivo*.²²²

Metal Coordination. Biopolymers, including silk fibroin,²²³ that contain pendent sulfhydryl (thiol) groups can associate with metal species. Silk fibroin modified with thiol groups rapidly gels upon adding gold (Au^{3+}), with the solidification occurring immediately after adding 5 mM Au^{3+} .²²³ Though transition metals can be cytotoxic, good cytocompatibility with the Au-SF hydrogels was observed, even with the highest Au^{3+} concentration.

3.1.4.2. Dynamic Covalent Interactions. Reversible covalent bonds are becoming more commonly employed for biopolymer modification due to their ability to form stable bonds that may later be reversed to liberate molecules by exposure to specific stimuli or to create self-healing materials.²²⁴ One example is the Diels–Alder reaction, where a typical form involves the reaction of a furan group with a maleimide group at a relatively low temperature (e.g., 50 °C) and the reversing of the reaction at a higher temperature (e.g., 150 °C).²²⁵ The use of the Diels–Alder reaction is increasing in popularity due to its mild reaction conditions and thermoreversibility²²⁶ and has been demonstrated on chitosan (Figure 2B),^{227,228} and alginate.²²⁹ Another example is the Schiff base bond formed between amino and carbonyl groups (i.e., aldehyde or ketone) and has been used for a variety of dynamic hydrogel materials, including chitosan.^{230–232} A recent review discusses these materials in more detail.²³³

Grafting From (GF) Strategies. The use of naturally derived dithiolanes, such as α -lipoic acid, can be used to create

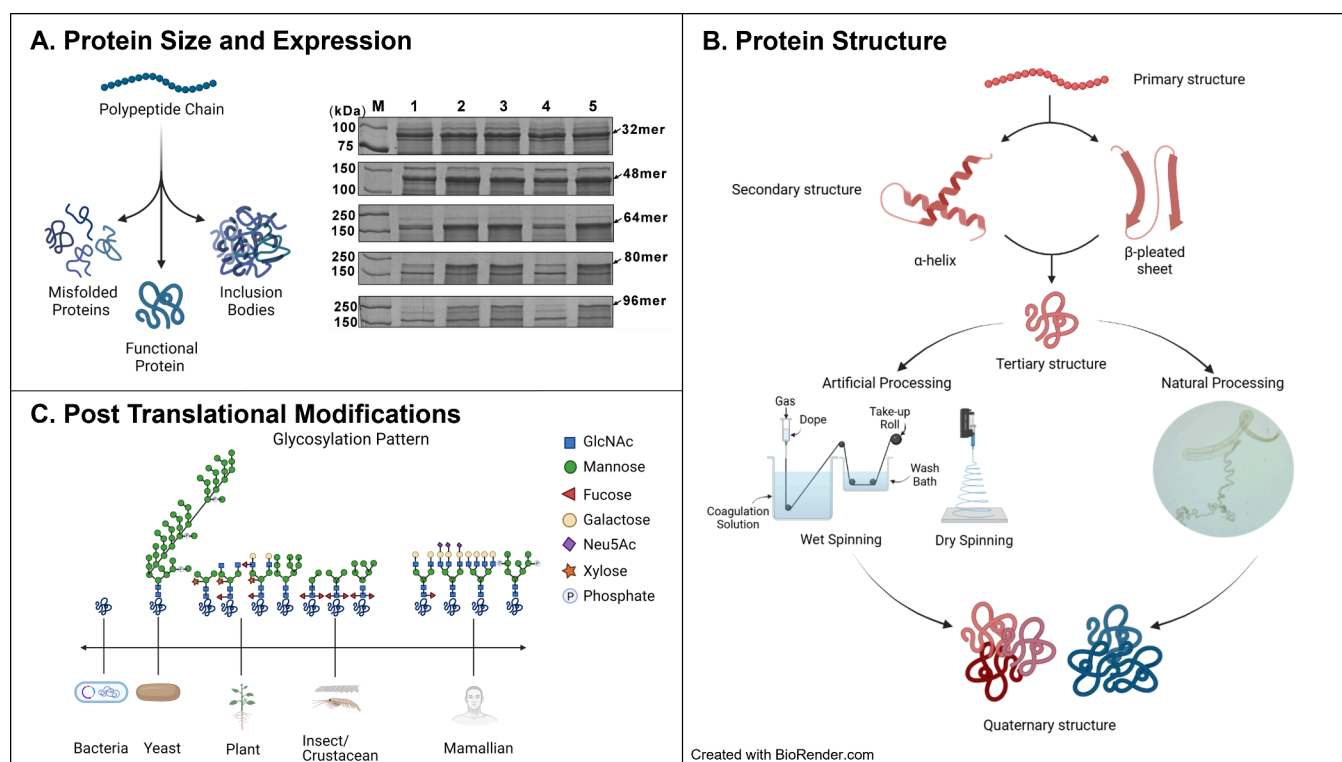


Figure 3. Biopolymers produced through recombinant methods have limitations and fail to mimic proteins produced by the native organism. A) Limits of bacterial production exist for larger proteins (<300 kDa), resulting in impaired protein folding and the formation of inclusion bodies. Low expression levels or truncation of the desired protein (spider dragline silk) are observed in the SDS-PAGE analysis as molecular weight increases in *E. coli*.^{3,4} B) Native biopolymers, such as silk fibroin, undergo natural processing, such as fiber spinning, where specialized organs (e.g., silk glands) process the fibers through physiological changes to assemble protein molecular structures that produce favorable mechanical properties.⁴ Fibers can be artificially spun through wet and dry spinning techniques, though the as-spun fibers often show weak mechanical properties and require post spinning treatments.^{4,7} C) Post-translational modifications such as glycosylation can be vastly different among commonly used expression hosts and native sources. These modifications can lead to a variety of issues with biocompatibility and protein folding, potentially producing an inferior product in an alternative source.^{8,9} Schematics were created with a license from BioRender.com and the Western blot shown in (A) was reproduced with permission from reference 3, ©2010 National Academy of Sciences.

reversible thiol/disulfide bonds. The 1,2-dithiolanes react with sulfhydryl groups to create protein–polymer conjugates or modified biopolymers with a wide range of potential applications. In synthetic systems, 1,2-dithiolanes can be used to create dynamic covalently cross-linked materials with self-healing properties.^{234–236} The thiol/disulfide exchange within these systems enables the development of materials with reversible disulfide bond formation. Composite keratin-based materials have been formed by these reversible reactions between sulfhydryl groups on the keratin with the dithiolane structure of α -lipoic acid.¹⁸²

3.2. Recombinant Protein Expression and Genetic Engineering. Over the last 45 years, technologies for the recombinant production of proteins have enabled the production of a wide variety of proteins, including engineered biopolymers and peptides, in non-native organisms such as bacteria and yeast, including protein therapeutics, antibodies, and biopolymers or partial biopolymer peptide sequences. The development of recombinant protein expression systems enabled the production of naturally derived biopolymers in non-native systems. Using the ever-improving array of gene editing technologies, investigators can not only produce the original protein but can engineer altered versions⁸² of the native sequence. The growing significance of these expression vehicles in present and future protein-based biopolymer manufacturing lies in their ability to provide a certain degree

of precision in controlling the molecular weight and sequence of the expressed protein and the resulting separation, purification, and reconstitution.^{13,14,82,152,237–245} Recombinant expression techniques have facilitated the rational design and assembly of essential functional motifs derived from natural biopolymer protein sequences across a spectrum of host systems, including bacteria, yeast, insects, mammalian cells, transgenic plants, and transgenic animals.^{246–248}

One of the main challenges that plague the production of modified biopolymers via recombinant expression is the successful production of sufficient quantities of large molecular weight proteins. Large biopolymer-based proteins that can be used to form biomaterials or drug delivery vehicles are high molecular weight (MW, > 200 kDa) with complex secondary structures, making them challenging to manufacture (express, purify, concentrate) at an industrial scale in common microbial systems.²⁴⁹ Additionally, although the rational design of numerous coding sequences is achievable, the translation to predicting the final protein architecture remains less straightforward but is a growing area of research with advancements in artificial intelligence and predictive machine learning algorithms.^{250,251} Moreover, confirmation of appropriate secondary and tertiary structures often necessitates extensive modification of the glycosylation pathways within the expression host or the utilization of complex processing

parameters to better mimic the native tertiary structure of the desired protein.²⁵²

3.2.1. Bacterial Expression Systems. Bacterial vectors are exceptional candidates for the expression of various proteins as they offer fast growth rates, comparably low costs, genetic stability, established protocols for molecular modification, and comprehensive knowledge of their biology.^{246,253} Notably, the bacterium *Escherichia coli* (*E. coli*) stands out as the most employed production system where its prevalence stems from several advantages, including its simplicity in purification stemming from the limited secretion of native proteins.²⁴⁷ Relevant to this review, sections of spider silk spidroin,^{3,13,254–257} keratin,^{258,259} resilin-like polypeptides (RLPs),^{15,153,260,261} and mucin-like polypeptides^{172,262} have been produced in *E. coli*-based hosts. As an example, RLPs, have been generated with tunable mechanical properties leading to a wide applicability across multiple tissue types within the musculoskeletal and cardiovascular systems.²⁶⁰ In 2007, Kim and colleagues showed that use of a lactose-induced *E. coli* fermentation method led to over 300 mg RLP/L of culture,¹⁴ leading to a stable, reproducible source of RLPs for use in materials fabrication.¹⁵

However, for many large and highly repetitive biopolymers such as *B. mori* silk fibroin, limiting factors, such as the inability to create proper glycosylation and post-translational modifications, metabolic constraints on the host cell, lack of physical property similarity between the generated proteins and their native counterparts, and insolubility of resulting materials due to formation of inclusion bodies, hamper translation (Figure 3).^{17,253,256,263} Investigators are still working to optimize expression rates, enhance protein secretion and purification, generate appropriate post-translational modifications, trigger desired secondary and tertiary protein structures, and/or achieve desired mechanical properties.^{13,15–17,264–269} Outside of *E. coli*, other bacterial expression systems have been used, to overcome challenges, improve protein secretion, or improve the economics of the recombinant silk production pathway. Recently, the Koffas group produced spider silk peptides in *B. megaterium*,¹² a species known for producing large flocculation proteins. Through a coculture system, they showed the degradation of biomass and the production of spider silk.¹² Similarly, in a collaboration led by the Zha group, the Koffas and Zha teams showed that a modified *Pseudomonas aeruginosa* (*P. aeruginosa*) strain could both use depolymerized polyethylene as a carbon source and then produce spider silk-like peptides.²⁷⁰

3.2.2. Yeast Expression Systems. Yeast expression systems have become valuable hosts to produce recombinant proteins with *Komagataella pastoris* (formerly known as *Pichia pastoris*) being the second most used system for protein biopolymer production, only behind *E. coli*.²⁷¹ These systems allow for eukaryotic post-translational modifications including disulfide bond formation and N-glycosylation which are crucial for proper protein folding and functionality. Additionally, they offer endotoxin-free production,²⁷² fast growth rates, and relatively simple genetic manipulation.²⁷³ These systems have been used to produce recombinant spider silk,²⁷⁴ mucin-like glycoproteins,²⁷⁵ and elastin-like polypeptides (ELPs).²⁷⁶ Yet, the native high-mannose-type sugar chains and glycosylation patterns in yeast can represent a biocompatibility constraint when introducing the products for biomedical applications and can limit the potential uses.²⁷⁷ Therefore, much effort has been put forth into *K. pastoris* and *Saccharomyces cerevisiae* yeast

expression vectors to produce therapeutic glycoproteins that display human-like glycosylation patterns,^{277–281} with successes in the biopolymer space with humanized mucin-like glycans.²⁷⁵ However, these alterations often come at a cost to the host system. For instance, the *K. pastoris* humanized mutant strain, Glyco4, exhibits impaired growth rates and cell wall defects. Zhu et al.²⁸² successfully identified a substantial cause of the observed phenotype, attributing them to a decreased expression of the glycosylphosphatidylinositol (GPI)-anchored cell wall glycoprotein, PpSpi1. Through upregulation of PpSpi1 in the new mutant strain, Glyco5, investigators were able to partially rescue the normal growth rate and cell wall integrity.²⁸² Although significant progress has been achieved in the humanization of yeast glycosylation patterns, further optimization remains a necessity for future applications in the production of biopolymers for medical applications.

3.2.3. Plant Expression Systems. Transgenic plants represent an underrepresented expression system where large-scale agriculture can be leveraged for the potential cost-effective creation of various recombinant biopolymers.²⁸³ However, currently the downstream processing to extract the protein of interest is greater than traditional systems hindering the realization of cost-effective production.²⁸⁴ To address this concern researchers have used directed expression, driving recombinant protein accumulation within specific tissue types, to streamline and mitigate this challenge.^{285–287} Currently, transgenic plants and plant cell systems have been utilized to produce an array of biopolymers including spider silk-like proteins,^{89,248} resilin-like polypeptides,²⁸⁸ elastin-like polypeptides,²⁸⁷ and mucins.²⁸⁹ As a growing expression system, plant and plant cell culture-based systems hold promise, but their commercial translation will be a key focus of both academic and industrial efforts in the coming years.

3.2.4. Insect Cells. Insect cell line expression systems are a promising candidate for the production of recombinant biopolymers, largely owing to the success of the baculovirus expression vector systems.²⁹⁰ Among the most widely utilized insect cell lines are S2, derived from *Drosophila melanogaster*, SF9 from *Spodoptera frugiperda*, and High Five from *Trichoplusia ni* with the majority of commercial applications focused on vaccine production with FDA approval for FluBlock²⁹¹ and Cervarix.²⁹² An advantage of insect cell line expression systems stems from their cost effectiveness in culturing conditions, characterized by the absence of CO₂ requirements and lower culture temperature, which is often near or at room temperature. These systems also facilitate the production of more complex glycan structures and post-translation modifications (Figure 3C). However, the non-mammalian glycosylation pattern inherent in insect cells may trigger an immunogenetic response in medical applications. To address this limitation, glycoengineering can facilitate production of mammalian-like terminal sialylated N-glycans in insect cells.^{293,294} These advancements in the humanization of glycosylation patterns position insect cells to generate recombinant proteins that closely mimic those produced in mammalian cell lines, but at a reduced cost and with reduced biosafety requirements. Predominantly, insect cells have been employed for vaccine production in the medical domain, however, noteworthy successes in biopolymer production have been made with partial sequences of *Araneus diadematus* dragline silk proteins ADF3 and ADF4 in Sf9 cells,²⁹⁵ a 70 kDa eGFP-spider silk fusion protein in BmN cells from *B. mori*

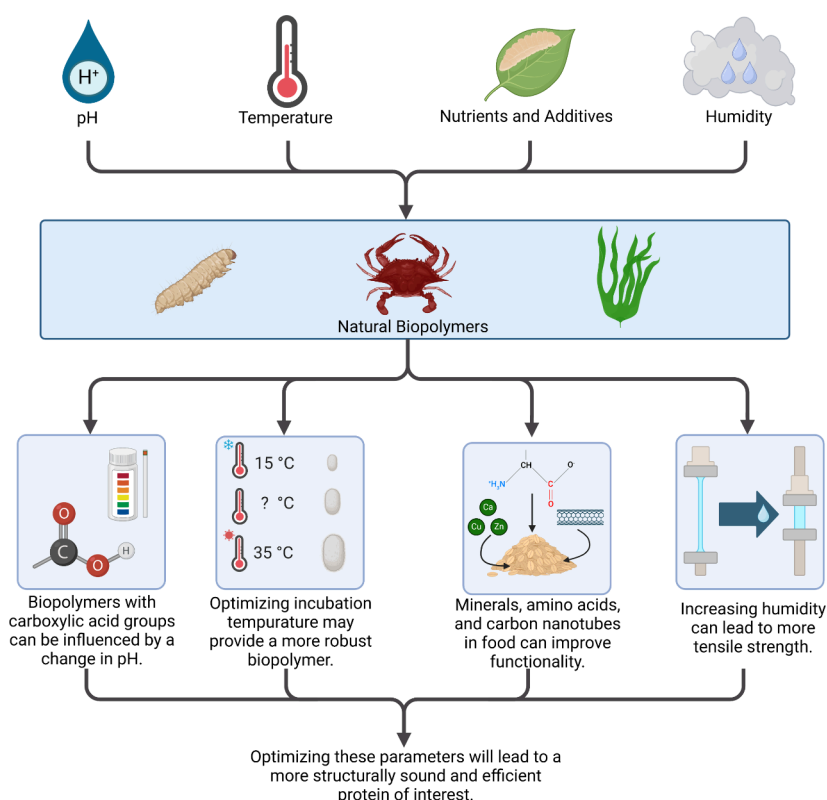


Figure 4. Influence of environmental factors on biopolymer production. Environmental factors play a crucial role in determining how organisms produce biopolymers and the quality of the resulting materials. While this variability poses challenges to standardizing sourcing processes, it also offers opportunities to modify properties and enhance the functionality of biopolymers. By understanding and manipulating these environmental factors, investigators can optimize biopolymer production for specific biomedical applications, leading to the development of novel and tailored biomaterials with enhanced properties. Schematics in this figure were created with a license from [BioRender.com](https://www.biorender.com).

silkworms²⁹⁶ and various collagen formats.²⁹⁷ Despite these advancements, the potential of this expression system to create mammalian-like biopolymers is still in its infancy, and its capabilities are yet to be realized.

3.2.5. Silk Gland Expression Systems. Genetically modifying organisms to utilize them as bioreactors or biofactories to produce recombinant proteins is a novel strategy to produce or enhance native biopolymers compared with traditional recombinant protein synthesis methods. One beneficial aspect of altering the native host species for utilization as a recombinant protein bioreactor is the decrease in engineering demand required to formulate the protein into its naturally found architecture. As an example, synthetic silk fibers must be artificially spun through a variety of methods (Figure 3B), including electrospinning, wet spinning, dry spinning, self-assembly, and microfluidics,^{298–300} with large scale production only recently realized through optimization by the Rising Research Group,^{13,301–304} located at the Karolinska Institutet (KI). These methods, recently reviewed from a variety of perspectives,^{298–300,305,306} utilize an array of solvents, coagulants, draw ratios, and physical parameters (such as varying pH, temperature, viscosity, voltage, polypeptide molecular weight, and blending with other organic molecules or metal ions) to produce the desired mechanical properties. However, while being useful for the tunability of the desired recombinant protein and its structures, these approaches require specific expertise and often results in fibers with altered mechanical properties compared to the naturally spun fibers from the silkworm or spider.

Thus, harnessing the silk gland for the production and self-assembly of various biopolymers offers an intriguing alternative to traditional recombinant expression systems. This organ, responsible for silk production, storage, and secretion, demonstrates notable efficiency in the production and self-assembly of biopolymers across a wide variety of silk fiber producing insects and spiders.^{85,307,308} Silk glands exhibit an extraordinary capacity to store very unstable proteins at high concentrations, preventing aggregation or denaturation, before secretion into the silk fibers.^{86,309} Therefore, the silk gland has great promise to produce full-length, highly repetitive protein biopolymers that currently challenge microbial-based expression hosts. Subsequently, current research has shown transgenic silkworms to be quite capable bioreactors for their high levels of expression and efficient secretion of numerous recombinant proteins. The *B. mori* expression system has demonstrated its capability to synthesize a wide range of proteins relevant to biomedical uses, such as human type III procollagen,³¹⁰ human lactoferrin,³¹¹ human serum albumin,³¹² human neurotrophin-4,³¹³ human epidermal growth factor,³¹⁴ and antibodies.³¹⁵ However, more relevant to the focus of this review, this system has also been employed to produce spider silk mimetics^{90,92,316} and silk fibroin-like fusion proteins.^{317,318} These silk fibroin-like biopolymers have modulated the mechanics or biological activity of the native biopolymer, highlighting the capacity investigators have in the rational design of future biopolymers using this platform.

The challenges encountered by silk gland expression systems include impurities stemming from the abundant production of

native silk fibroin support proteins (sericins) within the gland and incorrect folding of the desired protein primarily due to simpler glycosylation patterns found in insects compared to mammalian systems (Figure 3B).³¹⁹ Additionally, insect glycosylation patterns can induce a nonfavorable immune response in mammals. If left unaltered, this could limit the utilization of silk gland-produced biopolymers, as most therapeutic glycoproteins necessitate the more intricate mammalian type N-glycans for clinical effectiveness (Figure 3C).³²⁰ These challenges have been addressed through several bioengineering approaches. Knockouts of native silk genes have resulted in *B. mori* strains with empty silk glands³⁰⁹ resulting in a purer, naturally produced form of the desired protein.

Additionally, genome editing techniques also provide the means to improve the immune response when the biopolymer is introduced into humans. Utilizing a *piggyBac* vector and more recently CRISPR-Cas9 genome editing has shown the ability to alter the insect primitive N-glycosylation pathway to the more complex mammalian pathway.³¹⁹ This has been accomplished by conducting knockouts of critical biosynthetic genes in the host insect and then supplanting the glycosylation pathway with mammalian-appropriate genes transformed into the host genome.

3.3. Role of the External Environment in Biopolymer Structures. The easiest way to obtain a similar biopolymer with slightly different properties is to collect, purify, and evaluate that same biopolymer from a different host or native source. For example, the alginate community recognizes that alginates collected from similar species of sea kelp can yield polysaccharides with varying ratios of M and G units. Similarly, silk fibers from other moths or spiders produce silk fibers with mechanical and structural characteristics that are different from those of *B. mori*.^{53,303} However, not all naturally sourced biopolymers have readily available production alternatives. Thus, another way to alter protein structure is through modifying the environment where the host species produces the protein. One can think about this as the way they think about their hair. Keratin, the main protein in human hair and nails, responds to many things including the human's diet, the temperature and humidity in the environment, and the human's level of hydration.³²¹ Even things like hormone levels (e.g., pregnancy) can impact keratin production in humans.³²² Similarly, environmental factors can influence protein structures and production levels in other native host systems (Figure 4).

3.3.1. Known Impact of Climate and Climate Change on Natural Biopolymers. Naturally sourced materials are susceptible to environmental and climate changes, which can profoundly impact the physiology of organisms and consequently affect the downstream sourced product (Figure 4). A notable example of this influence is evident in the gradual warming and acidification of ocean waters due to increased carbon dioxide levels.³²³ The adsorption of CO₂ decreases the pH of ocean waters, diminishing the availability of calcium carbonate crucial for the formation of exoskeletons in crustaceans³²³ and increasing seaweed vulnerability to physical forces.³²⁴ Moreover, variations in light quality can significantly influence growth rates and metabolic pathways, thereby affecting the characteristics of the resulting product. For instance, temperature, photoperiod, and light quality greatly affect the growth, reproduction, and biomass content of algae.³²⁵ Similarly, cold-blooded animals, which are reliant on

external temperatures to regulate their body heat show large variation to varied conditions. Silk-producing organisms like spiders and silkworms are sensitive to temperature and rainfall fluctuations, which not only impact their growth rates but also influence the availability of essential food sources such as mulberry leaves for *B. mori*.^{326,327} These fluctuations can lead to a reduction in the quantity of biopolymers produced and alter the properties of the resulting fibers. Additionally, crustaceans also face varied growth rate.³²⁸ While this variability can pose challenges, it can also be strategically leveraged to regulate or modulate production or induce desired changes in biopolymer characteristics.

Few investigators have closely examined how the inherent biological regulation mechanisms found in the native producers can be harnessed for improved performance of biopolymers for biomedical uses. A significant constraint in leveraging these fluctuations is the logistical challenge of rearing a sufficient quantity of the native producer under controlled environmental conditions in an economically viable manner to obtain the necessary amount of biopolymer. We anticipate engineers and scientists can leverage environmental parameters such as the pH of the growth environment or water supplied, the temperature of the organism's growth environment, and the nutrients provided to influence biopolymer production. In a few areas, work has started to explore the role of external environmental cues on biopolymer structure and function.

3.3.2. Impact of Environmental pH Shifts in Biopolymer Production and Properties in Its Native Host. Biopolymers containing carboxylic acid groups, such as alginate and keratin, can be modified by changing the pH of the external environment to alter binding affinities and protein structure. For example, in the laboratory, the pH of water in shrimp rearing vessels can influence total chitin production and the thickness of shrimp shells.³²⁹ However, investigators did not monitor or investigate if there were shifts in chitin sequence or levels of acetylation. Moving forward, simple environmental changes could be used to modulate chitin or chitosan biopolymer sequences, potentially influencing key features such as degradation rates, rates of hydrolysis, solubility, and overall biopolymer production rates (Figure 4). Other proteins produced in hydrated or water-based environments such as alginates and aquatic silks, would also be hypothesized to be influenced by the pH and general ionic strength of the water.

3.3.3. Impact of Rearing and Production Temperatures and Humidity on Biopolymer Production and Properties in Its Native Host. Temperature and humidity play a crucial role in the metabolic pathways of many organisms and can cause changes to the biopolymer of interest. Ectothermic animals, such as crustaceans, insects, and arachnids rely on the outside temperature to regulate many processes within their bodies, leaving them predisposed to high variance in production which can be leveraged to modulate desired biopolymers. To understand these effects on the silking process for *B. mori*, Offord et al.³³⁰ systematically analyzed the influence of temperature and humidity on this process. These findings highlighted a reduced fiber diameter of 25% from larvae reared at 15 °C compared to 35 °C, morphological changes to cocoon structure, and modified tensile behavior. Interestingly, similar effects were observed in the phylogenetically distant silkworm species *Plodia interpunctella*. Shirk et al.³³¹ demonstrated that not only temperature had an influence on silk production, but also highlighted how population dynamics within the species

can regulate silk production. Furthermore, properties from orb webs were studied that inhabited a wide range of climate conditions in Colombia.³²⁷ Their findings showed spiders in regions with heavy rainfall had silk fibers with higher tensile strength and toughness compared to other groups, hypothesized to be a controllable mechanism by the spiders to receive less damage to their webs. Additionally, temperature can influence not only the expression of the primary silk protein components but also the glycoprotein components that affect the viscoelastic properties of the fibers.³³²

3.3.4. Impact of Nutrients and Additives on Natural Biopolymer Production in Its Native Host. Modification of natural protein structure, mechanical properties, and expression can be achieved through food additives in the diet of an organism. The formation of keratins in bovine claws is highly dependent on nutrient availability to epidermal cells involved in keratinization. Specific amino acids (Cys, His, and Met), vitamins (A, D, E, biotin), and minerals (Ca, Zn, Cu, Se, Mn) are essential for the activation of enzyme systems in keratinization, regulation of protein production, and structural and functional integrity of keratins.³³³ In *B. mori* silkworms, the addition of nanoparticles, metallic ions, and amino acid solutions to their diet alters the thermal and mechanical properties of silk fibers and impacts the secondary structure of the silk fibroin protein (Figure 4).³³⁴ Higher potassium content in silk fibers modified by the addition of tyrosine and fibroin amino acids (a mixture of hydrolyzed *B. mori* silk fibroin amino acids) increased the beta-sheet content of fibers, producing higher crystallinities, tensile strength, and strain energy density.³³⁴ Carbon nanotubes have also been investigated as a diet additive for their ability to improve the mechanical properties of the silk produced by spiders and silkworms. Mechanical properties of spider silk were significantly impacted after direct feeding of carbon nanotubes to spiders, including an increase in fracture strength from 1.5 to 5.4 GPa and toughness modulus to 1570 J g⁻¹ from 150 J g⁻¹.³³⁵ Similar results of improved mechanical, thermal, and electrical properties were observed when silkworms were fed a diet with carbon nanotube additives.^{336–338}

4. PERSPECTIVES ON FUTURE ADVANCEMENTS

Given its historical significance and renewed attention, naturally derived biopolymer therapeutics show immense potential. However, despite this rich tradition, only a limited number of biopolymers have been effectively utilized. In this review, our focus was on silk, chitosan, chitin, keratin, resilin, and mucins. It is crucial to recognize that even within these subsets of biopolymers, the field has yet to fully exploit the extensive biodiversity available for potential use. The primary hurdles in harnessing the diversity of biopolymers present in nature for new commercial product development in our opinion are 2-fold. First, sourcing the natural biopolymer in a scalable and economically feasible manner is only available for certain proteins and within certain species. An example relevant to this review is native resilin. While discovered in the 1960s,¹⁴² and characterized as one of the most efficient elastomeric proteins, its limited quantity found in various arthropod sources does not present itself to allow for enough material to be collected to make for a viable option for material formulation. It was not until the development of the recombinantly expressed RLP, rec1,³³⁹ in 2005 that allowed for the sourcing of naturally inspired resilin, paving the way for the biomaterials community to fabricate the diverse materials

highlighted in this review. Furthermore, optimizing purification strategies for the wide range of biopolymers presents a significant hurdle. A universal approach that accelerates the rapid processing of new biopolymers does not exist. Instead, each novel biopolymer necessitates meticulous processing evaluation to ensure that the protein of interest remains unaltered and that contaminants are absent in the isolated materials. Second, a multitude of factors, including biological diversity and environmental fluctuations, contribute to batch-to-batch variability in naturally derived biopolymers. This variability poses a significant challenge in applications where reproducibility is imperative. As we contemplate the future of sourcing reliable biomaterials, it becomes essential to consider and evaluate the potential impact of global warming and natural seasonal fluctuations on the biopolymer of interest. This aspect warrants critical attention, as it is an area that the biomaterials community has thus far dedicated relatively little focus to.

While challenges persist in sourcing naturally derived biomaterials, researchers have devised and investigated strategies to address these limitations. The predominant approaches for enhancing naturally occurring biopolymers involve chemical modification and recombinant expression of the target protein. Chemical modification offers a pathway to enhance the functionality of naturally derived biopolymers with established sourcing methods. However, the inherent chemical structure of the native material imposes limitations on the extent and location of chemical reactions. Furthermore, the harsh conditions required for chemical modifications, including temperature, pH, and solvents, can compromise the integrity of the native structure. As a result, while chemical modification may introduce additional functionality, it often comes at the expense of maintaining the original structural properties. Further advancement of strategies capable of functionalizing less reactive residues under milder conditions, such as the azo-reduction reaction targeting tyrosine residues,⁶ can help mitigate these limitations and need to be investigated further to expand the chemical modification potential of biopolymers.

Recombinant expression offers precise control over the genetic sequence of the biopolymer being produced and can help facilitate reliable sourcing of the desired biopolymer. However, the choice of expression organism significantly influences the downstream material properties, governing factors such as protein folding, molecular weight, and production yield. While impressive individual strides have been made in creating chemical modification strategies to modify native biopolymers and the creation of biomimetic recombinantly expressed proteins, future exploration in utilizing higher-order organisms, such as insects and plants, poses an exciting alternative expression platform to traditional microbial-based systems. Additionally, the integration of deep-learning models to advance the rational design of biomimetic materials^{340,341} shows promise in expanding novel recombinant biopolymers. These tools may eventually be able to accurately predict how various protein and polysaccharide structures react to certain stimuli. Enhancing the stability of a proteins or accurately predicting the biodegradation of a biopolymer. Reducing the number of iterative experiments, money, and time that is currently required to create novel biopolymers. Furthermore, as the field progresses it will be intriguing to observe how well the models predict self-assembly in alternative host expression systems.

The final tool highlighted in this review is precise control over the environmental factors that dictate the biopolymer of interest production. This is an underreported variable in the biomaterial community, but one that needs to be considered as we aim to improve standardization. While maintaining consistent environmental conditions is logistically challenging for most organisms to scale, understanding how different conditions can influence the downstream material will allow for better predictions of how the materials will behave. As we strive to expand the structural architecture of naturally derived biopolymers to create novel therapeutics, it is important to recognize that the methods for improving these materials discussed in the review are not mutually exclusive. While they each have practical limitations, they can be synergistically leveraged to enhance their efficacy. For example, one could envision strategically placing reactive residues within a recombinantly expressed protein to enhance its cross-linking capacity. To usher in the new wave of novel naturally derived and inspired biopolymers, a multidisciplinary approach needs to be adopted, providing the biomaterials community with consistent and effective natural materials.

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Notes

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