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Bioproduction of biomacromolecules for antiviral applications

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The societal damage brought on by viral epidemics indicates that next-generation antiviral treatments must be developed and deployed. Biomacromolecules are a diverse class of compounds that can potentially exhibit potent antiviral activity. Their efficacy and mechanisms of action are dependent upon multiple structural factors, including molecular weight, degree and position of sulfation, and backbone stereochemistry. Extracting biomacromolecules from animals and plants for healthcare applications is undesirable, as these methods are unable to yield products with well-defined chemical structures. Modern advances utilizing recombinant microbes and metabolic pathway engineering can be a key step towards large-scale bioproduction of tailored biomacromolecules for targeted antiviral applications.

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Introduction

In light of the severe economic and mortality costs of recent viral epidemics, such as COVID-19, MERS, and SARS, rapid development and production of molecules with antiviral activity is a desirable goal. Many biomacromolecules, including poly-γ-glutamic acid, glycosaminoglycans, mucin, chitosan, and algal polysaccharides, are able to inhibit the infectivity of a broad range of viruses through diverse mechanisms [1,2]. The biocompatibility of these compounds combined with their broad-spectrum antiviral activity suggests that therapeutics based on these biomacromolecules can potentially be deployed against novel viruses in much shorter time frames compared to traditional vaccines [3]. Modern advances in metabolic engineering and recombinant production will facilitate continued investigation and development of these materials for antiviral applications by allowing large-scale production of well-defined and complex biomacromolecules. This review will discuss recent advances in the bioproduction of antiviral biomacromolecules in the context of challenges facing large-scale manufacturing of products with high therapeutic activity.

Antiviral activity of biomacromolecules

Poly-γ-glutamic acid (γ-PGA), glycosaminoglycans (GAGs), mucin, chitosan, and algal polysaccharides have been shown to inhibit viruses *in vitro* and *in vivo*. Table 1 summarizes the antiviral activity of these biomacromolecules, including the types of virus studied, notes on biocompatibility, and mechanisms of action. These biomacromolecules can be highly efficacious in inhibiting viral infection. For example, researchers explored the effects of administering 30 μl of 1 mg/mL 3000 kDa γ-PGA intranasally to mice 12 hours prior to infection with a lethal dose of influenza A virus subtype H5N2. It was found that 100% of mice receiving the γ-PGA treatment survived with minimal symptoms, while the mice in the control group had a 0% survival rate [4].

Research also suggests that the chemical structure of antiviral biomacromolecules significantly impacts efficacy. Table 2 summarizes findings regarding changes in antiviral activity resulting from alterations in molecular weight (MW), backbone composition and stereochemistry, and degree and position of sulfation. For example, a 2019 study found that increasing the molecular weight of heparin from 4.1 kDa to a range of 6−14 kDa decreased 46-fold the concentration of the biomacromolecule needed for 50% inhibition of cellular binding by human adenovirus type 37 [5]. Thus, structural parameters represent a toolbox through which engineers and researchers can use bioproduction to enhance the antiviral properties of these biomacromolecules. In the following sections, we present a discussion of recent advances in the bioproduction of several classes of antiviral biomacromolecules, emphasizing the challenges faced in scalable synthesis of homogeneous well-defined constructs which can potentially exhibit enhanced activity.

Poly-γ-glutamic acid

Poly-γ-glutamic acid (γ-PGA) is a polypeptide produced and secreted by various *Bacillus* bacteria and consists of repeating D-glutamic or L-glutamic acid residues linked via α-amino and γ-carboxylic acid groups (Figure 1). γ-PGA exerts antiviral activity by upregulating the host organism's initial immune response to a virus, specifically

Table 1

Antiviral activity of selected biomacromolecules

Macromolecule	*In vivo* inhibition	*In vitro* inhibition	Biocompatibility	Mechanism of Action	Ref.
γ-PGA	Influenza A H5N2, Influenza A H1N1, Norovirus	SARS-CoV, HCV, HSV-1, HSV-2	**High biocompatibility**. No cytotoxicity in *in vitro* studies. For *in vivo,* γ-PGA is tolerated in high amounts as a food additive. No discernable side effects from oral or intranasal administration.	Upregulation of host organism/cell immune response, leading to increased cytokine production.	[1,4,6–8]
GAGs	HIV-1, CMV, Dengue, Flaviviral encephalitis	SARS-CoV-2, Influenza A H1N1, Adenovirus, HSV-1, HSV-2, Dengue, Orthopoxvirus	**Moderate to high biocompatibility**. No cytotoxicity in *in vitro* studies. For *in vivo,* often no side effects observed. However, oral and intramuscular GAG usage in humans has been documented to cause some side effects, including gastrointestinal discomfort.	Competitive binding to viral particles. Often involves anionic sulfates binding to cationic viral capsid regions.	[9–14, 15–17,18]
Mucin	Influenza A H1N1, Rotavirus, Coronavirus (TGEV), Enterovirus (EV70)	Influenza A H1N1, HIV-1, Norovirus, Rotavirus, Poxvirus, HPV, MCV	**High biocompatibility**. No cytotoxicity in *in vitro* studies. For *in vivo*, mucin is naturally consumed by humans (milk), and found ubiquitously in the body. No observable side effects from oral administration.	Competitive binding to viral particles. Involves sialic acid residues binding to cationic viral capsids and spike proteins.	[19–21, 22–27]
Chitosan	Influenza A H7N9, Influenza A H1N1, Influenza A H9N2, Influenza B, NDV	HIV-1, RLV, HPV	**High biocompatibility**. No cytotoxicity in *in vitro* studies. For *in vivo*, non-toxic and commonly consumed (seafood). Side effects of pharmaceutical/supplement administration are rare and mild, including mild gastrointestinal discomfort.	Upregulation of host organism/cell immune response, and competitive binding to viral particles via anionic sulfate groups.	[28–32]
Algal Polysaccharides (APs)	Mumps, Influenza B, Influenza A H1N1, HIV-1, RSV, PIV, Rhinovirus, MPV, Coronavirus OC43 and 229E, HPV, Yellow Fever	SARS-CoV-2, Dengue, HPV, HIV-1, Influenza A H1N1, HSV-1, HSV-2, CMV, Yellow fever	**Moderate to high biocompatibility**. *In vitro* studies show a range of toxicities dependent on the type of AP used. For *in vivo*, APs are FDA approved as a food additive. No side effects for nasal administration. Oral administration rarely causes gastrointestinal discomfort.	Competitive binding to viral particles via anionic sulfate groups. Evidence exists for various secondary mechanisms including inhibition of RNA reverse transcriptase	[2,33,10, 34–39]

by increasing cytokine production (Table 1). Oral and intranasal administration of γ-PGA is effective against multiple virus types and free of side effects. Important factors impacting the antiviral activity of γ-PGA (Table 2) include molecular weight and backbone stereochemistry, which can be controlled with recently developed metabolic engineering techniques. Microbial production of γ-PGA from biomass is well established, and currently used for commercial production in the food, medical, and wastewater industries [50]. While past achievements in this space have increased the yield and efficiency of γ-PGA production, demonstrating titers as high as 101 g/L, contemporary innovations involve precisely manipulating stereochemistry and molecular weight [51]. In 2019 Halmschlag *et al.* recombinantly introduced multiple forms of γ-PGA synthetase and glutamate racemase genes into *Bacillus subtilis* in a modular fashion, thus producing γ-PGA with a molecular weight ranging between 40 and 8500 kDa and stereochemistry from 3%–60% D-glutamate depending on specific combinations of recombinant genes [52•]. Similarly, researchers showed that γ-PGA produced by strains of

Table 2

Structural features affecting antiviral activity

Macromolecule	Molecular weight	Backbone residue composition	Backbone stereochemistry	Degree of sulfation	Stereochemistry of sulfation	Ref.
γ-PGA	X		X			[1,6–8]
GAGs	X	X		X	X	[2,9,5, 12–14,39–43]
Mucin	X	X				[19–21,23–27]
Chitosan	X	X		X	X	[33,29,31,44,45]
Algal Polysaccharides	X	X		X	X	[2,33,39,46–49]

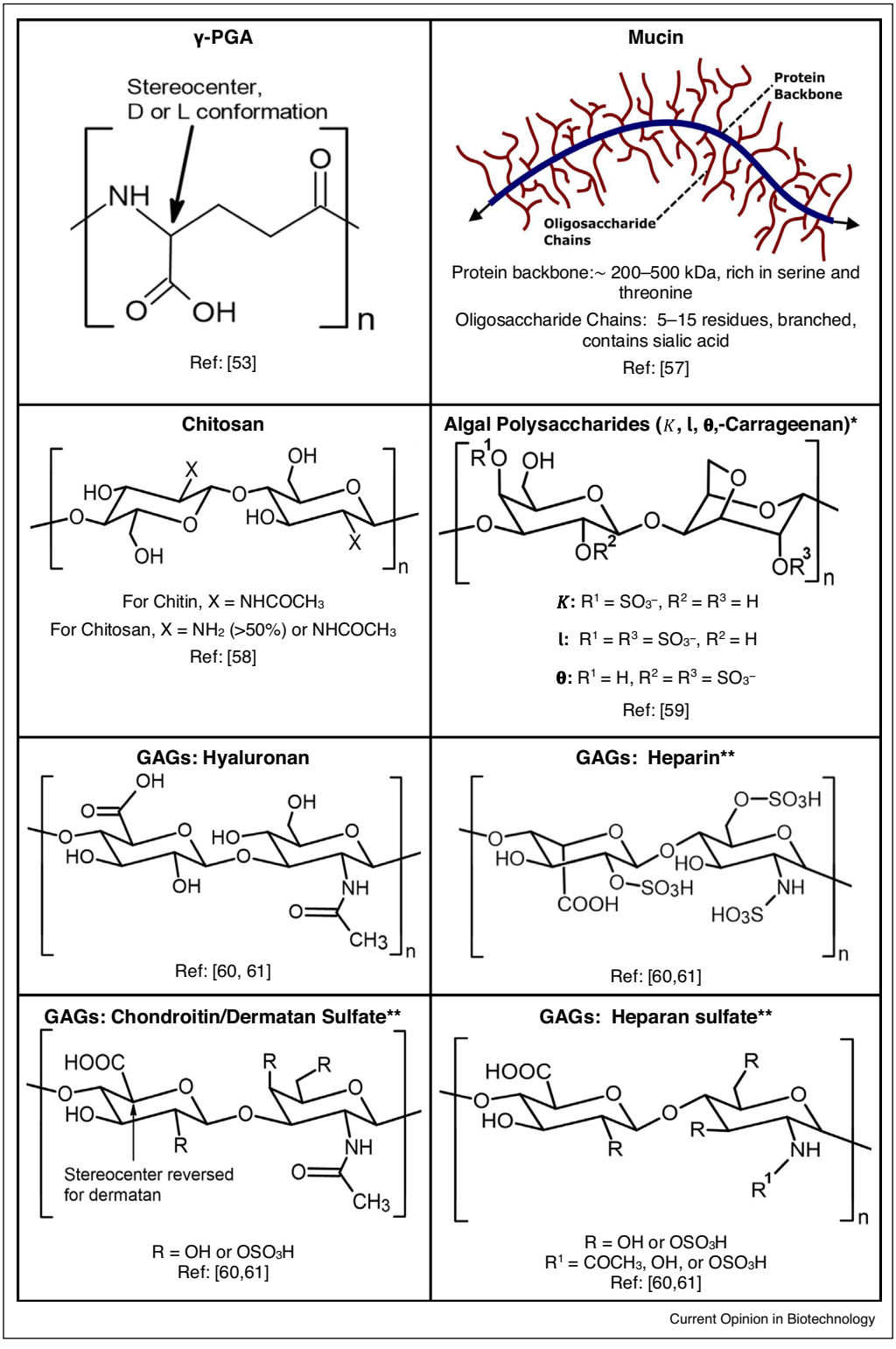
Corynebacterium glutamicum with various forms of recombinant γ-PGA synthase genes subject to adjustable promoter strength could produce γ-PGA with a customizable L-glutamic acid content ranging from 97.1%–36.9% and a molecular weight range of 2000 kDa–4000 kDa [53•]. A related work by Sawada *et al.* observed a change in the D/L stereochemistry ratio from 75/25 to 5/95 when the *pgsA* gene in *B. subtilis* was knocked down [54•]. Additional works have continued to explore the modulation of γ-PGA molecular weight. Feng *et al.* found that by knocking out the genes *upp*, *pgdS*, *ggt*, and *cwlO* in various combinations, the molecular weight of 328 kDa γ-PGA can be tuned from −9.1% to +36%. Work by Scoffone *et al.* found deleting *pgdS* and *ggt* genes in *B. subtilis* increases molecular weight from 240 kDa to 490 kDa [55,56]. Table 3 summarizes these advances in bioproduction of γ-PGA as well as the other biomacromolecules discussed, including the most promising methods, species utilized, and structural control achieved. Further development of γ-PGA as an antiviral therapeutic will require systematic studies of relating structure to antiviral activity to understand which specific stereochemistry and molecular weight should be targeted for maximal efficacy.

Glycosaminoglycans

Glycosaminoglycans (GAGs) are integral components of vertebrate cell surfaces, intracellular compartments, and extracellular matrices [41]. Generally, they are linear anionic polysaccharides with a backbone consisting of repeating disaccharide units, which are frequently sulfated (Figure 1). GAGs, namely hyaluronan, chondroitin/dermatan sulfate, heparin, and heparan sulfate, display antiviral capabilities primarily through competitive binding to viral capsids and spike proteins. Efficacy is chiefly dependent on molecular weight, degree and position of sulfation, and backbone composition (Tables 1 and 2). Because extraction of GAGs from animal tissues is unsustainable, prone to contaminations, and yields heterogeneous products, the most promising route for obtaining GAGs with well-controlled chemical structure at large scales involves recombinant microbes (Table 3). While recombinant production of hyaluronan has existed at laboratory scale for over 13 years, Cheng *et al.* recently achieved titers suitable for large-scale operations by using genome-scale metabolic pathway analysis in *C. glutamicum* to yield 28.7 g/L of hyaluronan [62•]. However, it should be noted that microbial production at this level may be hindered by the high viscosity of hyaluronan, a problem that may be addressed through the examination of bioproduction factors such as novel microbial hosts, altered extracellular environments, and production of precisely sized hyaluronan [63]. Researchers have also demonstrated molecular weight control, with one study using a glucostat during fermentation with a *Lactococcus lactis* stain containing recombinant *has*-operon genes. The strain produced hyaluronan with a monodisperse molecular weight ranging from 0.4 MDa to 1.4 MDa, which was dependent on the glucostat setpoints and the specific combination of *has*-operon genes used [64].

Novel developments in the recombinant production of chondroitin and dermatan sulfate have also been recently demonstrated. Both of these biomacromolecules exhibit antiviral activity (Table 1), but until recently, there was no documentation of successful recombinant production. In 2019, Badri and coworkers were able to produce animal-free chondroitin sulfate for the first time by employing recombinant chondroitin-4-sulfotransferase and utilizing metabolic engineering to achieve accumulation of the universal sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) [65••]. Moreover, Tykesson *et al.* were able to produce recombinant and defined dermatan sulfate (a GAG closely related to chondroitin sulfate) for the first time. This study also demonstrated the ability to generate dermatan sulfate with novel sulfation patterns, including a homogeneously sulfated IdoA-GalNAc-4S polymer and its 2-*O*-sulfated, 6-*O*-sulfated and 2,6-*O*-sulfated derivatives [66••]. Recombinant production of heparosan, a precursor for heparin and heparan sulfate, was also recently demonstrated by introducing the *Pasteurella multocida* heparosan synthase gene into recombinant *Bacillus megaterium*, which led to simultaneous synthesis of low and high molecular weight heparosans (10 kDa–300 kDa) at up to 2.74 g/L in fed-batch cultivations [67•].

Figure 1



The chemical structure of selected antiviral biomacromolecules, Refs. [53•,57–61].
*Because of the structural diversity of algal polysaccharides (carrageenans, fucoidans, alginates, etc.) only a few pertinent morphologies of carrageenan are included. Readers are directed to Ref. [59] for a more in depth look at the various structures of algal polysaccharides.
**Variation exists in the sulfation pattern and/or backbone residues of these biomacromolecules. Readers are directed to Refs. [60,61] for more information.

Table 3

Advances in bioproduction of antiviral biomacromolecules

Macromolecule	Promising methods	Species utilized	Notable titers	Structural control achieved	Ref.
γ-PGA	Metabolic pathway engineering on species which naturally produce γ-PGA, often using genetic knockdown.	*B. subtilis*, *C. glutamicum*	Up to 101 g/L	MW[a]: from 40 to 8500 kDa. Stereochemistry: 2.9%–60% D-glutamate.	[51,52•,53•,54•,55,56]
GAGs	Recombinant production w/ or w/o metabolic pathway engineering. Control of fermentation conditions. Oligosaccharide synthesis.	*C. glutamicum*, *L. lactis*, *E. coli*, *B. megaterium*	HA: 28.7 g/L, Heparosan: 2.74 g/L	HA MW: from 0.4 MDa to 1.4 MDa CS: % sulfation controlled, from 0.31–36.9 DS: 3 different sulfation patterns achieved HP and HS: Multiple sulfation patterns and MWs achieved for oligosaccharides (using recombinant enzymes).	[60,63,64,65••,66••,67•,68]
Mucin	Recombinant production of mucin-like glycopeptides.	CHO, 293-F, breast cancer cells, *E. coli*, *S. cerevisiae*	100 mg/L	Differences in sialic acid content and glycosylation sites among products from human cell lines. Site-specific glycosylation on protein backbone achieved in *E. coli*.	[23,69•,70–72]
Chitosan	Optimized fungal fermentations. Recombinant production using heterologous enzymes and exogenous monomers. Oligosaccharide synthesis.	Multiple fungal species, *S. cerevisiae*, *E. coli*	2.3 g/L, 138 g/kg dry biomass, 3–5 mg/L heterologous enzyme[b]	Control over the degree of deacetylation (70–90%), solubility, MW, and polarity.	[73–76]
Algal Polysaccharides	Metabolic pathway engineering on species which naturally produce algal polysaccharides. Including downregulation of pathogenicity.	*P. aeruginosa*	1 mL/mL per OD_{600}	Ability to target a β-D-mannuronic acid to α-L-guluronic acid ratio of 80:20.	[77]

[a] Abbreviations: Molecular Weight (MW), Hyaluronan (HA), Chondroitin sulfate (CS), Dermatan sulfate (DS), Heparin (HP), Heparan sulfate (HS).
[b] Titer's are not reported as an amount of biomacromolecule produced, but as an amount of heterologous enzyme capable of synthesizing the biomacromolecule.

Compared to γ-PGA, the bioproduction of GAGs with high titers and tailored chemical features is lagging due to their more complex chemical structures. Future research enabling recombinant GAGs to be used for antiviral applications should focus on increasing titers, especially for chondroitin and dermatan sulfate, as they have only been expressed in small quantities to date. Additionally, heparin and heparan sulfate show antiviral efficacy (Table 1) but have not yet been produced recombinantly and can thus be a promising area of future exploration. Moreover, more work should be done to understand the antiviral efficacy of GAG oligosaccharides, which can potentially be produced with exact weights and sulfation patterns more easily than full-length GAGs [68].

Mucin

Mucins are extracellular glycoproteins that can be secreted or membrane bound [58]. Mucins have a 'bottle brush' structure with a protein backbone (~200–500 kDa) comprising up to 60% serine, threonine, and proline repeats, and oligosaccharide chains attached to the protein core consisting of 5–15 monomers, including sialic acid (Figure 1). Studies have implicated binding of mucin's sialic acid residues to viral capsid proteins as the key mechanisms through which mucin exerts antiviral activity against several classes of viruses, including coronavirus, HIV-1, and influenza H1N1 (Table 1) [20–22,80]. Mucin can be harvested from natural sources, such as pig stomachs, but these methods are low-yielding (3.57 g mucin/stomach), resource intensive, and produce heterogeneous forms of mucin [69•,79,80]. This suggests that recombinant production may be a promising strategy for large-scale production of medical-grade mucin. Because of its large size and complex post-translational modifications involving oligosaccharides, large-scale mucin bioproduction is in a more preliminary stage compared to γ-PGA or GAGs [69•].

In 2019, Shurer *et al.* were able to produce a full-length recombinant mucin protein (MUC1), complete with

extensive *O*-glycosylation, using 293-F cells with a codon-optimized MUC1 gene [69•]. Additionally, researchers have capitalized on the relative ease of production of miniature mucin-like glycopeptides, which can also show robust antiviral activity [24]. By introducing heterologous enzymes, such as human *N*-acetylgalactosaminyltransferase, into yeast, Amano *et al.* produced recombinant mucin-like glycopeptides that were approximately $^{1}/_{4}$ the size of native mucin [70]. Truncated mucin peptides (approximately $^{1}/_{3}$ of native size) can be produced in CHO cells at 100 mg/L per day, indicating progress towards large-scale bioproduction [72]. Likewise, structural control has been achieved through the production of a recombinant protein in *Escherichia coli* with site specific incorporation of a mucin-like glycan by evolving a new tRNA aminoacyl synthetase-tRNA pair [71]. Recombinant production of truncated mucin-like glycopeptides has seen the most success and is a promising pathway for the development of mucin as an antiviral therapeutic (Table 3). However, much work remains in understanding the antiviral activity of truncated mucin or mucin-like constructs compared to full-length mucin.

Chitosan and algal polysaccharides

Chitosan, the deacetylated form of chitin, is a linear polysaccharide that forms the exoskeleton of crustaceans and is comprised of β-1-4 linked D-glucosamine and *N*-acetyl-D-glucosamine units [81]. Algal polysaccharides, including carrageenans, agarans, alginates, fucans, and ulvans, help form the cell walls of marine algae and seaweed [34]. The antiviral mechanism for both chitosan and algal polysaccharides involves binding of the anionic sulfate groups of these biomacromolecules to positively charged regions of viral capsids and envelopes (Table 1). Chitosan can exert additional antiviral activity through immunostimulation, including upregulating cytokine and lymphokine production [81–83]. Because these biomacromolecules are abundant in nature, current production of chitosan and algal polysaccharides mainly utilizes abiotic extraction methods. However, these methods yield heterogeneous products, lead to overharvesting and ecological damage, and can produce concentrated alkaline waste streams [59,84].

Progress in the recombinant bioproduction of these materials represents one of the most promising directions for producing well-controlled medical grade marine polysaccharides. Through the utilization of several fungal species' natural ability to produce chitosan, optimization of fungal fermentations is a front-runner for the industrial-scale bioproduction of chitosan with precise size and structure. This is accentuated by the fact that up to 60% of biotech industries already use fungi in processes such as antibiotics and enzyme production [73]. Kim *et al.* achieved production of 2.3 g chitosan/L when *Absidia coerulea* was grown under optimized conditions involving a pH 4.5, agitator speed of 250 rpm and aeration rate of 2 vvm [74]. Likewise, fermentation of the fungi *Gongronella butleri* has been used to achieve up to 138 g chitosan/kg dry biomass [73]. Importantly, the use of different fungal species and fermentation conditions can yield consistent structural differences in the chitosan produced, including a percentage deacetylation that ranges from 70−90% [73]. Factors such as pH, addition of transition metals, and media composition, are indicated as key foci for future work exploring the efficient bioproduction of precisely structured chitosan via fungi. Notwithstanding, other species are being investigated as well, with the recombinant production of chitin and chitosan oligosaccharides being demonstrated in yeast cells by expressing chitin synthase 2 and supplying the cells with β1,4-linked *N*-acetylglucosamine monomers. Including varying amounts of *N*-propanoyl glucosamine, *N*-butanoyl glucosamine, and *N*-glycolyl glucosamine affected the ratio of chitin to chitosan and allowed control over the solubility and polarity of the chitosan [75]. Likewise, Martinez *et al.* produced mutated chitinolytic enzymes D200A and D202A in *E. coli* and showed that these formerly chitin-degrading enzymes became effective chitosan polymerization agents after mutation, exhibiting the ability to catalyze a transglycosylation reaction on chitosan oligosaccharides when provided with excess chitosan or chitosan-like monomers [76].

Bioproduction methods have also shown promise for algal polysaccharide production. *Pseudomonas aeruginosa* is one of only a handful of bacterial species with the native capability to synthesize alginate, but scale up of the process is infeasible due to the bacterium's intense pathogenicity. However, Valentine *et al.* have demonstrated the overproduction of alginate in a non-pathogenic *P. aeruginosa* strain via the overexpression of the *mucE* gene, and the downregulation of five key pathogenicity genes, thus, laying groundwork for scaling the bacterial bioproduction of alginates. Moreover, the alginates produced were similar to those derived from seaweed in terms of monomer composition and rheological properties [77]. Additionally, the entire pathway for algal polysaccharide production in the algae genus *Saccharina* was recently characterized. The pathway begins with fructose-6-P as a substrate and involves the action of over a dozen unique enzymes, whose identities and functions were confirmed through their cDNA isolation, recombinant production, and subsequent activity assay [78]. Equipped with this pathway knowledge, future work can look to produce recombinant well-defined algal polysaccharides with enhanced antiviral efficacy and aim to achieve sufficient titers for large-scale biomedical applications. Since they are easier to recombinantly produce, systematic studies examining the antiviral activity of chitosan oligosaccharides in comparison to native full-length versions should be performed. Moreover, only a small number of recombinant hosts have been tested for recombinant marine

polysaccharide production so far (Table 3). The genetic diversity of other common recombinant hosts outside of yeast and *E. coli* (*Bacillus* sp., mammalian cells, transgenic plants, etc.) implies that some may be more efficient at producing marine polysaccharides. Experimentation with a greater range of recombinant hosts can potentially increase the titer and structural diversity of marine polysaccharides for antiviral applications.

Conclusion

A robust body of literature demonstrates that biomacromolecules such as γ-PGA, GAGs, mucin, and marine polysaccharides, can exhibit potent antiviral activity. These biomacromolecules can be potential tools in the fight against global pandemics caused by viral pathogens. Traditional extraction methods for these biomacromolecules dominate current markets, however, these methods lack sustainability at large production scales, are prone to contamination, and can result in ecosystem destruction. Furthermore, the products obtained by traditional extraction suffer from structural heterogeneity. Thus, metabolic pathway engineering and recombinant production can be a superior strategy for manufacturing antiviral biomacromolecules at large scale. Using recently developed methods, well-defined chemical structures with enhanced antiviral activity can be targeted and sustainably synthesized (Table 3).

In order for biomanufactured antiviral biomacromolecules to become a therapeutic reality, future work will need to address three main challenges: maximizing titers, synthesizing biomacromolecules with well-defined structure, and understanding the relationship between structure and antiviral activity. Larger titers may be obtained by experimenting with a variety of host systems, and will help address the drawback of high price that exists for many biomanufactured therapeutics. Well-defined chemical structures will require creative metabolic engineering strategies. The production of truncated or mimetic polypeptides may be beneficial in addressing both titer and structural challenges. Lastly, the ability to create biomacromolecules with homogeneous, defined structures will enable researchers to gain a more systematic understanding of the relationship between structure and antiviral efficacy. Through continued progress in these arenas, biomacromolecular antiviral therapies may be developed as a key medical response to future viral pandemics.

Conflict of interest statement

Nothing declared.

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