Review

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Bacteriophage-derived depolymerases as antimicrobial synergists: A strategy to overcome resistance

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SUMMARY: Upon infection, bacteria form polysaccharides barriers, such as capsular polysaccharide (CPS), exopolysaccharide (EPS) and lipopolysaccharide (LPS). The barrier hinders antibiotic penetration and host immune clearance, exacerbating antimicrobial resistance crisis. Bacteriophages (phages), natural viruses that can specifically infect and kill bacteria, have evolved depolymerase to degrade the polysaccharides. This review evaluates the primary therapeutic value of depolymerases as synergists to existing therapies, systematically detailing their potential to enhance antibiotic efficacy, improve phage therapy, and augment host immunity. We further integrate an evolutionary perspective to analyze likely adaptive responses and potential strategies to eradicate resistance. Finally, the discussion addresses formulation challenges and future prospects for the clinical translation of depolymerase-based synergistic therapies.

Keywords: bacteriophages, depolymerase, biofilm dispersal, polysaccharide, antibacterial reagent, antibiotic resistance reversal, combination therapy, resistance evolution

1. Introduction

Bacteriophages (also known as phages) are viruses that infect microorganisms such as bacteria, fungi, algae, actinomycetes or spirochetes. With the largest number and highest genetic diversity in the earth's biosphere, phages can be found in every explored biome, and their number is estimated to be up to 10^{31} (*1*). Their proliferation strictly depends on the host: the process begins with injection of phage genomes into the host, and the genome destroys bacterial metabolism, eventually lysing the bacteria (*2*). Phages have become an effective alternative for antibiotics, especially with the increasing prevalence of multidrug-resistant (MDR) bacteria worldwide.

Phage infection begins with the critical step of the phage selectively attaching to the bacterial surface receptors, often mediated by tail fiber or tail spike proteins (TSPs) on the phage. Then adsorption is achieved through receptor binding protein (RBP), a prerequisite for cell wall degradation (*1,3*). This highly-specific process allows phages to inhibit bacterial growth with minimal effects on accompanying microflora of a target bacterium during infection (*4*). This precise targeting, however, is often hampered by the presence of polysaccharides, such as capsular polysaccharide (CPS), exopolysaccharide (EPS) and lipopolysaccharide (LPS), on the bacterial surface. Similarly, disruption of phage action occurs when the bacteria live as biofilms, a barrier preventing from the phage adsorption and penetration (*5*). The physical barrier also impedes drug penetration and masks bacterial surfaces, thereby conferring antibiotic resistance and impeding host immune recognition (*5,6*). Phage have evolved depolymerases that degrades bacterial polysaccharide to facilitate phage access (*4*).

There is increasing research attention that targets phage-derived depolymerase as a novel and powerful antimicrobial agent. Previous reviews have comprehensively cataloged their diversity (*4*), biochemical activities, and therapeutic efficacy (*7-9*), with particular emphasis on their potency in biofilm control (*10-13*). However, emerging evidence suggests that the most significant therapeutic value of depolymerases may not lie in their direct antibacterial activity, but in their ability to act as synergistic agents that enhance antibiotic efficacy and immune responses. While rarely inducing classic bacterial resistance problem (*14*), their therapeutic

application will inevitably exert evolutionary pressure, potentially driving bacterial adaptive strategies.

Therefore, this review expands from a functional summary of depolymerase to discuss a novel framework that integrates synergistic potentiation and evolutionary machinery. The discussion first establishes the role of depolymerases as antimicrobial synergists, detailing how their activity potentiates antibiotics, augments phage therapy, and empowers host immunity. Then, we address the related evolutionary dynamics, from potential bacterial resistance pathways to counterstrategies, such as protein engineering and combination therapies. By integrating the perspectives of synergistic therapy and evolutionary machinery, this article provides a next generation rationale for developing depolymerases into human therapeutics.

2. Overview of phage depolymerases

Phage depolymerases were partially purified from phage solutions by Hughes *et al.* in 1998 (*15*). As follows, the basic characteristics of phage depolymerases have been elaborated. These foundational understanding is critical for translating their function into practical application, such as synergistic therapeutics and engineered solutions.

2.1. Depolymerases play an important role in bacterial infection

CPS, EPS and LPS are decorative polysaccharides on the surface of bacterial cells. CPSs are found on the cell surface of a variety of bacterial species, binding tightly to the cell surface in a covalent attachment. As the protective component of pathogenic microorganisms, CPSs facilitate host colonization and physically prevent the action of bactericide by forming a barrier on bacterial surface (*16,17*). EPSs are the main matrix that enclose the bacteria cells in the biofilm mode of life (*18*). The significant difference between bacteria in biofilms and their planktonic counterparts is that biofilm protect bacteria from environmental stress conditions, such as chemicals, antibiotics, and immune cells attacks. Biofilms increase the resistance of some bacteria against the conventional antibiotics by around 1000-fold (*19,20*). Unlike CPSs, EPSs are not tightly bind to the cell and are often secreted into the extracellular matrix (*21*). LPS is usually considered as a ligand for phage tail fiber proteins during adsorption to the host bacteria (*4*). In addition, LPS also plays an important role in protecting bacteria from anti-bacterial peptides (*22*). Collectively, polysaccharides can reduce the absorption efficacy of some phages, and they also play a critical role in bacterial virulence and biofilm production. Another critical role of polysaccharides lies within their ability to act as a physical barrier against the penetration of conventional antibiotics and the host immune system (*23-25*). This simple but potent defensive role makes degrading the polysaccharides a strategic target for both phage infection and antimicrobial therapy.

Many phages produce depolymerase that specifically binds and degrades bacterial surface polysaccharides. Unlike lytic phages that destroy the cell, depolymerases act with high precision, stripping away these barriers to expose the underlying bacterial cell surface. The exposure sensitizes bacteria to external threats, such as antibiotics and immune effectors.

2.2. Common phenotype of depolymerases: The halo effect

When phages with depolymerases form plaque on bacterial lawn, the plaque is usually transparent in the center and surrounded by a translucent halo. The halo will continue to expand during incubation, while the size of the clear plaque does not change, constituting a typical phenomenon of depolymerase activity. It occurs phage replication slows down when bacteria are in stationary stage, while the excess depolymerases can still degrade the extracellular polysaccharide components of bacteria (*7*). This visual hallmark is a key indicator for identifying and isolating depolymerase-producing phages.

2.3. Diversity of depolymerases

With the ongoing exploration of phage-derived proteins, depolymerases have revealed remarkable diversity. According to the work of Pires DP *et al.* (*7*), most of phage depolymerases are considered structural proteins, as they are either encoded by or found in proximity to phage structural genes (such as tail fibers, base plates, and sometimes also in the neck). A small number of depolymerases are considered as soluble proteins since their coding genes are located far from any structural genes.

Based on the mechanisms of breaking cell barriers, phage depolymerases were distinguished into two classes: hydrolases and lyases (*7*). Both enzymes possess the ability to degrade carbohydrate barrier. Hydrolases catalyze the hydrolysis of glycosidic bonds in CPS and O-antigen on LPS chain (*26*), and there are six subtypes: sialidases, levanases, xylosidases, dextranases, rhamnosidases, and peptidases (*7*). The lyases cleave (1,4) glycosidic bonds by a β-elimination mechanism without using water molecule, and they are comprised of five groups: hyaluronate lyase, pectate lyase, alginate lyase, K5 lyase, and O-specific polysaccharide lyase (*8*). This mechanistic diversity supports their ability to target a wide spectrum of chemically distinct polysaccharides critical for bacterial defense.

The difference between the mechanism of hydrolysis and β-elimination results in differences in substrate specificity, so that therapy must be formulated with deliberations (*27*). Hydrolases, such as sialidases, dextranases, cleave glycosidic bonds via

hydrolysis, generating saccharides. Their actions are crucial for degrading neutral polysaccharides such as dextran and levan, commonly found in biofilms of *Lactobacillus* or *Bacillus* (*28*). In contrast, lyases, such as hyaluronidases and pectate lyases, act on acidic polysaccharides containing uronic acids [*e.g.*, alginate in *Pseudomonas aeruginosa* (*P. aeruginosa*), hyaluronan in *Streptococcus*] through β-elimination, producing unsaturated oligosaccharides with a double bond at the non-reducing end (*28,29*). The difference in catalytic mechanism and product chemistry suggests a divergence in their synergistic efficacy. For instance, alginate lyases degrade the viscous alginate matrix of *P. aeruginosa* biofilms to improve phage or antibiotic diffusion (*30*), but the unique unsaturated oligosaccharide produced have been reported to exhibit immunomodulatory or biofilm-dispersing activities (*31*). Conversely, the "cleaner" breakdown by hydrolases like dextranases may remove a physical barrier without significantly altering the chemical microenvironment. Therefore, combination therapy strategy should be optimized with regard to the selection of a depolymerase (hydrolase *vs*. lyase) based on the chemistry of biofilm maximize efficacy.

The mechanistic dichotomy also indicates differing biophysical requirements for optimal activity *in vivo*. Lyases often require specific cations (*e.g.*, Ca^{2+}) as cofactors for β-elimination, and the optimal activity is achieved under a narrower pH range with respect to the pKa of the uronic acid substrate(*32*). While also pH-dependent, hydrolases tend to manifest versatility through larger pH range, but susceptible to end product inhibition (*33*). Understanding these nuances is crucial for formulating stable, effective enzyme cocktails for specific infection microenvironments, such as the acidic wound bed or the cation-rich respiratory mucus.

2.4. Structure and function of depolymerases

Despite their great diversity, depolymerases share conserved structural features. The majority of depolymerases are present as elongated homotrimers within the tail fiber or tail spike of the phage baseplate (*26*). However, there are rare exceptions: depolymerase of *Escherichia coli* (*E. coli*) siphophage 63D is characterized as a homotetramer (*34*). Depolymerases are fibrous proteins with a parallel β-helix topology, which includes the active sites that facilitate the recognition and binding of specific sequences in the surface polysaccharides. The complex structure of depolymerase underlies their high chemical stability, allowing them to remain active across a wide range of temperature and pH (*26,35*).

A canonical trimeric depolymerase, such as that from the deep-sea thermophilic phage GVE2, comprises three domains: an N-terminal domain connects to receptor-binding protein and the phage baseplate, a central domain responsible for substrate recognition and enzymatic activity, and a C-terminal domain crucial for the formation of trimers (*36,37*). This modular architecture separates structural attachment, catalytic function, and oligomerization, constituting the key feature that facilitates natural evolution and enables protein engineering. Historically, trimerization was considered essential for function (*38*), while recent studies, such as Kp34gp57 capsular depolymerase of *Klebsiella* phage crystalize as a monomer, is largely due to the intra-subunit active site formed by beta-barrel insertion domains (*39*). This expands the structural understanding and suggests flexibility for engineering simplified, monomeric variants.

The structure of depolymerases can be modified to adapt to a wide array of polysaccharide receptors in vertical and horizontal transfers. Vertical transfer involves mutating the central domain at protruding loops of β-helix in the active site without altering its overall conformation. The alteration enables the peptide chain to adapt to different polysaccharide substrates (*26*). The horizontal transfer essentially replaces the existing central catalytic domain with a foreign one, allowing the immediate acquisition of new host specificities. According to Latka, *et al.*, such horizontal structural adaptation is supported by ample evidence from phages CUS-3, HK620, Sf6, and P22 (*26*). This evolutionary plasticity highlights their potential for engineering. The conserved N- and C-terminal domains can serve as a stable scaffold, allowing the grafting of novel catalytic domains to create depolymerases with tailored or broadened specificity. Such engineering strategies are key to developing evolutionarily robust therapies.

3. Applications and implications of depolymerases

The application of phage depolymerases has evolved from utilizing existing enzymes into strategically integrating enzymes into combination therapies and engineered platforms (*14*). This section moves beyond their standalone functions, focusing on their core value in synergistic combinations and addresses the evolutionary dynamics of their application.

3.1. Depolymerases precisely eradicate biofilm formation

Phage depolymerases degrade polysaccharides on the host bacterial surface, facilitating the subsequent adsorption, infection, and disintegration of host bacteria. Evidence from diverse animal models, including *Galleria mellonella* (*40,41*), zebrafish (*42*), chicken (*43,44*), and mice (*45*), confirms their potential to eradicate bacterial infection and improve survival rates. Their activity is particularly potent against biofilms, as they degrade the EPS matrix that encloses and protects bacterial communities. Ample reports indicate that depolymerases can disrupt biofilms *in vitro* and confer high levels of protection *in vivo*, such as depolymerase Dp42 from

Klebsiella pneumoniae (*K. pneumoniae*) phage vB_KpnP_IME409 (*45*), Dep-ORF8 from *Pasteurella multocida* (*P. multocida*) phage PHB02 (*46*), and Dp49 from *Acinetobacter baumannii* (*A.baumannii*) phage IME285 (*47*).

The high specificity of depolymerases enables targeted action, with certain enzymes active against only a single capsular type (*48*). While this specificity restricts the utility of mono-enzyme therapy to a narrow spectrum, it also makes depolymerases ideal tools for bacterial typing and precise diagnosis (*26,48*). Consequently, the full therapeutic potential of these enzymes is realized not in isolation, but in concert with other antibacterial agents and host immune defenses. This diagnostic precise can, in turn, inform subsequent targeted combination therapies.

3.2. Depolymerase acts as a synergist

Depolymerases have immense potential as powerful synergists for antibiotic treatments, and their synergistic actions manifest in multiple ways (Figure 1). A well-documented synergy is the enhancement of antibiotic efficacy. Depolymerases enhance antibiotic efficacy by degrading CPS and EPS matrix of biofilms to facilitate contact between bacteria and treatment. Hence, the simplest synergistic effect of depolymerase is to promote the effect of disinfectants. Chai, *et al*. reported that the combination of a *Klebsiella* depolymerase and chlorine dioxide removed approximately 92% of the biofilm bacteria (*49*).

Likewise, similar synergies can lower the minimum inhibitory concentration (MIC), even empowering antibiotics that are ineffective otherwise. Notably, the depolymerase from *K. pneumoniae* phage KPO1K2 significantly enhanced the efficacy of ciprofloxacin against biofilms. The effects extents to enabling gentamicin which was ineffective against biofilms alone. Data indicate potent effect of KPO1K2 synergy by reducing bacterial counts by 3.261 log, with a reduction of 5.373 log in young biofilms (*50*). Studies on depolymerase Dpo71 further strengthens the paradigm, indicating Dpo71's ability to remove the capsular barrier and facilitate binding of colistin to the bacterial surface. Another study in *Galleria mellonella* infection model indicate that the combination of Dpo71 and colistin achieved an 80% survival rate, significantly outperforming either agent alone (40% and 30% survival, respectively) (*51*). Considering the novel studies and the limitation of conventional antibiotics, depolymerase pre-treatment sensitizes resistant bacteria to provide a key strategy to revitalize existing agents.

Beyond antibiotics, depolymerases function as natural synergists for lytic phages. Their enzymatic activity pre-conditions the bacterial surface by cleaving receptor-masking polysaccharides, therefore contributing to phage absorption. Studies indicated that a recombinant depolymerase can extend the host range of phages to otherwise resistant bacteria. Specifically, the recombinant depolymerase targeting the KL51 capsule of *Klebsiella pneumoniae* expands the host range (*52*). Furthermore, a combined application of *Staphylococcus aureus* (*S. aureus*) phage *Kayvirus rodi* and the polysaccharide depolymerase Dpo7 (from a different phage) better eradicated *S. aureus* biofilms than either agent alone (*53*). With this regard, engineered phages expressing single/multiple recombinant depolymerases represent a promising strategy for enhanced killing efficacy and broader host range for phage therapies.

Depolymerase action also synergistically alter the dynamics of immune responses, potentially presenting as immunoadjuvant strategy. Ample reports indicate that depolymerases can reduce bacterial virulence and sensitize them to the immune system (*45,54,55*). Mechanistically, this involves the removal of immunosuppressive polysaccharide, therefore exposing pathogen-associated molecular patterns (PAMPs) such as peptidoglycan, lipoproteins, and unmasked LPS core components. These exposed PAMPs are recognized by specific host pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), on innate immune cells. Immune

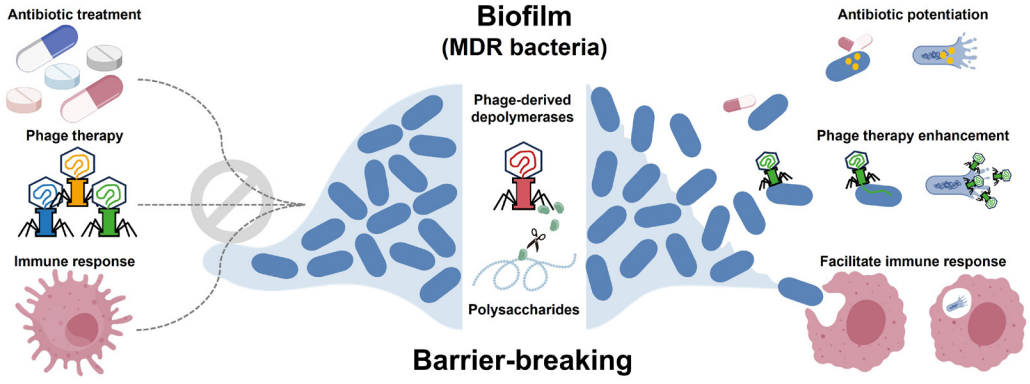

Figure 1. The potential of depolymerases as synergists. By enzymatically degrading key polysaccharide barriers (CPS, EPS and LPS), depolymerases remove a major obstacle to antimicrobial agents and immune effectors. Figure created with Biorender.

recognition triggers downstream pro-inflammatory signaling pathways (*e.g.*, NF-κB, MAPK), leading to the upregulated expression of cytokines, chemokines, and adhesion molecules. Consequently, depolymerase enhances opsonization and phagocytosis by macrophages and neutrophils (*56,57*), promoting a robust and coordinated recruitment of immune effectors to the infection site. In contrast with the antibiotic synergy, this synergy between depolymerase and host immune system presents an immunoadjuvant advantage of eliminating the bacteria without triggering widespread endotoxins release into the surrounding system, thereby reducing the scale of the infection and associated inflammatory events.

3.3. Bacterial resistance against depolymerase and potential counterstrategies

While depolymerases rarely induce classical resistance in the manner of antibiotics, their therapeutic use still exerts selective pressure that can drive adaptations. To design durable treatments, it is essential to anticipate these potential evolutionary routes. Key adaptations may include: a) Modification of the polysaccharide substrate (*e.g.*, altering acetylation (*58*)) to evade enzymatic recognition; b) Phase variation to express a different polysaccharide serotype resistant to the originally deployed depolymerase (*59,60*); and c) Upregulation of alternative EPS production to create a new, chemically distinct matrix (*61*).

These adaptive evolutions often emerge with exploitable fitness cost that jeopardizes the defensive integrity of bacteria and sensitize them to therapeutics. For instance, a point mutation and frameshift in the *wbaP* gene within the *cps* gene cluster of *K. pneumoniae* led to downregulated CPS production and upregulated production of smooth LPS. Though altering the glycocalyx profile, the evolutionary change attenuates virulence, enhances opsonization, and makes the bacteria more prone to immune clearance (*61*). This phenomenon reveals a strategic opportunity, in which depolymerase therapy can be designed not only to eliminate bacteria, but also to apply a selective pressure that actively steer bacterial populations towards a more vulnerable state to be exploited by a follow-up agent, such as antibiotic or the host immune system.

To preempt resistance, the application of depolymerase must be guided by evolutionary principles. Three main strategies can be employed to outmaneuver bacterial adaptation (Figure 2):

a. Cocktail Therapy: This approach utilizes mixtures of depolymerases that targets distinct polysaccharide antigens or epitopes on the same bacterium, making simultaneous escape mutations less probable. An example is phage ΦK64-1 which encodes 11 distinct depolymerase genes to counteract 11 different *Klebsiella* capsular types (*62*).

b. Genetic Engineering: Depolymerases can be engineered into multifunctional agents, such as depolymerase-lysin fusions, that attack independent, essential bacterial structures. Alternatively, protein engineering can broaden substrate specificity to target multiple variants.

c. Rational Combination Treatment: Depolymerases can be purposedly combined with antibiotics or phages in rotated or sequential therapy, with the depolymerase as a pioneer agent. This "depolymerase-first" approach aims to first strip away the polysaccharide shield, then immediately followed by a second agent (*e.g.*, a conventional antibiotic, phage, or other disinfectants) that exploits the newly exposed bacterial vulnerabilities. This creates a temporal elimination of two independent targets that sequentially takes out the external biofilm and the bacteria, making concurrent evolutionary escape difficult for the pathogen. This applies multifaceted selective pressures, reducing the chance that the pathogen evolves successful coping mechanism.

3.4. Delivery strategies and formulation of depolymerase-based therapies

Translating these synergistic and evolutionary concepts into antibacterial therapeutics requires novel delivery strategies and a thorough consideration of their biological fate as protein therapeutics. For topical treatments, delivery can be achieved by developing topical hydrogels or wound dressings for localized, sustained release of depolymerase into chronic wound infections resulted by bacterial biofilm. Infection in the respiratory system can be treated with direct delivery into the lungs with inhalable powders or spray drying (*63*). To further enhance stability and targeting, advanced formulation strategies such as microencapsulation within biodegradable polymers or encapsulation in liposomes (nanoliposomes) show great promise. These systems can protect the enzyme from premature degradation, control its release rate, and improve tissue-specific delivery while masking it from immune surveillance(*14,64,65*).

However, successful delivery hinges on addressing pharmacokinetic (PK) and safety of administered enzymes. These factors include *in vivo* stability, serum half-life, and immunogenicity upon administration. A critical aspect of immunogenicity is the risk of generating anti-drug antibodies (ADAs), which could intervene with the depolymerase activity and lead to hypersensitivity reactions that limits long-term therapeutic efficacy (*64,66*). While depolymerase action avoids the sudden endotoxin release associated with bacteriolysis, the rapid, large-scale degradation of structural polysaccharides could itself modulate local immune responses. The nature of this modulation remains an important area for preclinical safety assessment, as it could exacerbate inflammation. Their commercial viability is indicated by existing non-phage-derived EPS depolymerases,

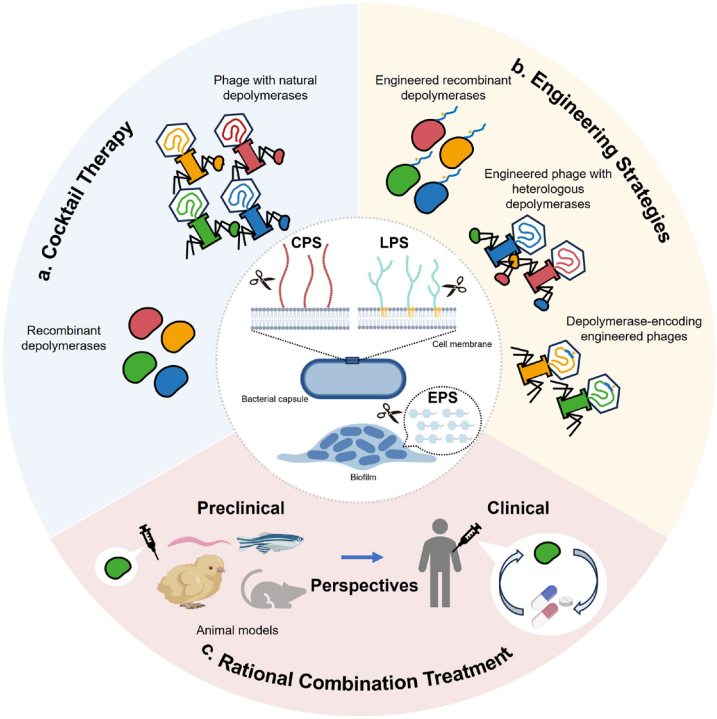

Figure 2. Schematic diagram of the counter-strategies of depolymerase as synergists. a) Cocktail Therapy: The foundational strategy employs mixtures of depolymerases targeting distinct polysaccharide antigens (*e.g.,* different capsule types) on the same pathogen. This multi-target approach increases the genetic barrier to resistance, making simultaneous escape mutations less probable. b) Engineering Strategies: A more advanced strategy involves direct protein engineering to create superior enzyme agents. This includes designing bifunctional fusion proteins (*e.g.*, depolymerase-lysin) that attack independent bacterial structures, or modifying the enzyme's active site to broaden its substrate specificity, thereby covering multiple pathogen variants with a single agent. c) Rational Combination Treatment: The most integrative strategy purposefully combines depolymerases with other antimicrobials (antibiotics or phages). Applying multifaceted selective pressures through rotation or sequence (*e.g.*, depolymerase first to strip the capsule, followed immediately by an antibiotic) hinders bacterial adaptation and prevents escape. Figure created with Biorender.

such as alginate lyase (Sigma-Aldrich) and dispersin B (Kane Biotech) (*7*), suggesting existing accessibility of enzyme-based biofilm control. Nevertheless, transitioning from commercial enzymes to human therapeutics demands rigorous evaluation under regulatory frameworks, requiring standardized assays for enzyme activity, potency, and stability in biologically relevant environments. Though developing existing enzyme-based products into clinically available products may require further research, existing evidence already indicate a clear path for future development. Therefore, the essence of future research lies within materializing the existing concept to develop an evolution-resistant combination therapy. With that regard, formulating depolymerase-based therapy is not a distant technical hurdle, but a critical breakthrough within reach.

4. Conclusion

As we approach a post-antibiotic era, finding sustainable alternatives to combat multidrug-resistant bacteria is at urgent need. The global threat of antibiotic resistance has led to tremendous interest in phage therapy (*67*). This review has synthesized current knowledge to propose that phage-derived depolymerases represent a unique class of antimicrobial adjuvants, manifesting potent synergistic functions. Our discussion elaborated on the depolymerases' ability to sensitize pathogens to conventional antibiotics and facilitate immune system eradication, therefore raising a concerted approach that mitigates the limitations of prior therapies.

While depolymerases rarely induce classic resistance (*67*), their use will exert selective pressure. Therefore, we believe future development of depolymerase-based therapies must focus on the evolutionary aspect of pathogens. Specifically, we proposed that cocktails therapies or other deliberate combinational treatments might manifest as effective counterstrategies. Although clinical application of depolymerases still require ample research, their proven efficacy in preclinical studies against biofilms and encapsulated bacteria is promising (*42,49,67,68*). A critical step towards the clinic will be navigating the unique regulatory pathway for depolymerases, which likely fall under the category of "antibiotic adjuvants" or novel enzymatic biology. This necessitates the development of consensus standards for defining and measuring enzymatic potency in therapeutic contexts, distinct from conventional antibiotics. In conclusion, depolymerases are likely ineffective as standalone replacements for antibiotics, rather as pivotal components of a synergistic and evolution driven strategy against bacterial infection. Further research

into the engineering, formulation, pharmacokinetics, immunogenicity, regulatory strategy, and integration of depolymerase into combination therapies is paramount to realizing their full potential in reducing our reliance on conventional antibiotics.

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