Journal of Pharmaceutical Analysis 14 (2024) 100906

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

journal homepage: www.elsevier.com/locate/jpa

Review paper

Contemporary strategies and approaches for characterizing composition and enhancing biofilm penetration targeting bacterial extracellular polymeric substances

Lan Lu [a, 1, *], Yuting Zhao [b, 1], Mingxing Li [c], Xiaobo Wang [d], Jie Zhu [a, **], Li Liao [a], Jingya Wang [a, ***]

[a] Key Laboratory of Medicinal and Edible Plants Resources Development of Sichuan Education Department, Sichuan Industrial Institute of Antibiotics, School of Pharmacy, Chengdu University, Chengdu, 610000, China
[b] Meishan Pharmaceutical Vocational College, School of Pharmacy, Meishan, Sichuan, 620200, China
[c] Laboratory of Molecular Pharmacology, Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou, Sichuan, 646000, China
[d] Hepatobiliary Surgery, Langzhong People's Hospital, Langzhong, Sichuan, 646000, China

ARTICLE INFO

Article history:
Received 4 August 2023
Received in revised form
8 November 2023
Accepted 26 November 2023
Available online 29 November 2023

Keywords:
Analytic strategies and approaches
Composition characterization
Extracellular polymeric substances (EPS)
Promoting biofilm penetration

ABSTRACT

Extracellular polymeric substances (EPS) constitutes crucial elements within bacterial biofilms, facilitating accelerated antimicrobial resistance and conferring defense against the host's immune cells. Developing precise and effective antibiofilm approaches and strategies, tailored to the specific characteristics of EPS composition, can offer valuable insights for the creation of novel antimicrobial drugs. This, in turn, holds the potential to mitigate the alarming issue of bacterial drug resistance. Current analysis of EPS compositions relies heavily on colorimetric approaches with a significant bias, which is likely due to the selection of a standard compound and the cross-interference of various EPS compounds. Considering the pivotal role of EPS in biofilm functionality, it is imperative for EPS research to delve deeper into the analysis of intricate compositions, moving beyond the current focus on polymeric materials. This necessitates a shift from heavy reliance on colorimetric analytic methods to more comprehensive and nuanced analytical approaches. In this study, we have provided a comprehensive summary of existing analytical methods utilized in the characterization of EPS compositions. Additionally, novel strategies aimed at targeting EPS to enhance biofilm penetration were explored, with a specific focus on highlighting the limitations associated with colorimetric methods. Furthermore, we have outlined the challenges faced in identifying additional components of EPS and propose a prospective research plan to address these challenges. This review has the potential to guide future researchers in the search for novel compounds capable of suppressing EPS, thereby inhibiting biofilm formation. This insight opens up a new avenue for exploration within this research domain.

1. Introduction

Bacterial biofilms (BF) are organized aggregates of microorganisms that reside in an extracellular polymeric matrix and are irreversibly attached to living or non-living surfaces; they are the leading cause of long-term, recurrent infections in hospitals [1,2]. The formation of bacterial biofilms is closely related to the production of extracellular polymers. Extracellular polymeric substances (EPS) are organic polymers of microbial origin responsible for linking cells and other particulate materials or adhering substrates together in biofilm systems [3], and are comprised of extracellular polysaccharides, proteins, nucleic acids, lipids, and other biomolecules, providing a three-dimensional (3D)-protected support for bacteria [4]. The matrix is rich in negatively charged compositions and hydrophobic groups, and the pores are filled with water, which facilitates the transport of nutrients [5].

As an integral component of all biofilms, the formation of EPS occurs during the initial phase of biofilm attachment to the surface. In essence, the formation of microbial biofilms on both living and

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: lulan@cdu.edu.cn (L. Lu), arsyn1116@gmail.com (J. Wang), purejie@qq.com (J. Zhu).
[1] Both authors contributed equally to this work.

https://doi.org/10.1016/j.jpha.2023.11.013

non-living surfaces is heavily influenced by the quantity of EPS produced. Consequently, the physical properties of EPS exert a significant impact on the characteristics and structure of biofilms. Moreover, the extracellular polymer matrix plays a predominant role in facilitating mutual microbial communications within the biofilm [6]. Bacteria embedded within the EPS exhibit the ability to transmigrate resistance genes, engage in synergistic interactions, and facilitate cell-cell communications [5]. Thus, mechanical properties such as viscoelasticity and cohesiveness of the EPS depend on the physicochemical properties of the biofilm polysaccharides, such as their molecular weight, radius of gyration, local concentration within the biofilm, and their interaction with other EPS constituents [7]. Furthermore, the constituents of EPS can obstruct specific cell and small-molecule assaults. Within the innermost layers of EPS, oxygen and nutrient availability is constrained, leading to the emergence of dormant persister cells. These persister cells expedite the development of antimicrobial tolerance and resistance, while also offering defense against the host's immune cells [8–10].

Hence, the components of EPS, which play pivotal roles in both the structure and function of biofilms, have driven contemporary researchers to explore superior and innovative compounds that can impede biofilm formation [11,12].

In this comprehensive review, we present an overview of the existing analytical methods employed for characterizing EPS compositions. Additionally, we discuss innovative strategies aimed at enhancing biofilm penetration by targeting EPS. A critical evaluation of the limitations associated with colorimetric techniques, specifically, is provided. Furthermore, we address the challenges inherent in identifying EPS components and propose a prospective research plan for their comprehensive characterization.

2. Primary components of the extracellular polymer matrix

The synthesis of major components of EPS, which include exopolysaccharides, fibrous and globular proteins (including extracellular enzymes), nucleic acids, and lipids, is systematically regulated by environmental stimuli for a variety of biological functions, including adhesion, protection, and energy storage [13]. Over 40 varieties of polysaccharides and proteins have been identified in *Bacillus subtilis* (*B. subtilis*), *Candida albicans* (*C. albicans*), *Escherichia coli* (*E. coli*), *Haemophilus influenzae* (*H. influenzae*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). The compositions and functions of EPS in biofilms are summarized in Table 1. As mentioned previously, polysaccharides are closely associated with structural integrity and protection, and play a significant role in maintaining EPS stability and structural integrity in biofilm formation, providing hydration and resistance to physical forces, and acting as a nutrient source and barrier against harsh environments, antibiotic penetration, and host immunity permeability [10,14]. Proteins play a fundamental role in governing various bacterial behaviors, including adhesion, motility, aggregation, mechanosensing, cell-to-cell binding, interactions with fungi, and the metabolism of essential components such as carbohydrates, amino acids, lipids, nucleotides, energy, vitamins, as well as degradation and remodeling of EPS [10].

In brief, EPS plays important roles in maintaining the 3D structure of the biofilms by acting as a “multifunctional scaffold” that protects embedded bacteria, building and maintaining a heterogeneous chemical and physical milieu, and providing nutrients for resident bacteria. Therefore, disrupting the constituents of EPS renders bacteria within biofilms more susceptible to antibiotics, leading to the eradication of mature biofilms [15].

2.1. Extracellular polysaccharides

The majority of biofilms are dependent on secreted extracellular polysaccharides, which are the primary component of EPS [4,16,17]. EPS provides essential foundations for the formation and endurance of biofilms, enhancing structural stability, offering physical and chemical protection against antimicrobials, facilitating microbial cell adhesion and aggregation, absorbing organic and inorganic compounds, and serving as a carbon source in nutrient-deficient environments [18–20]. Diverse bacteria are capable of producing versatile polysaccharides. Some are reserved in cells (such as glycogen), and others are secreted into extracellular space as dissociative polysaccharides (such as cellulose and alginate) that contribute to the formation of the biofilm substrates [14]. The formation of extracellular polysaccharides also sets the foundation for long-term and chronic infections, as cells embedded in biofilm are less likely to be eliminated by the immune cells of the host or antibiotics. For example, poly-β-(1,6)-N-acetylglucosamine (PNAG) is an extracellular polysaccharide that contributes to bacterial aggregation. This substance is known as a polysaccharide intercellular adhesin (PIA). Due to the importance of extracellular polysaccharides in the formation and maintenance of biofilm fabric, research has been conducted based on glycoside hydrolases for dispersing biofilms. It has been reported that nanomolar doses of the glycoside hydrolases PslGh and PelAh could inhibit and rapidly disperse biofilms by selectively targeting and degrading the exopolysaccharide components in *P. aeruginosa*. Using colorimetric analytical assays and confocal laser scanning microscopy (CLSM) imaging to detect specific staining, these two glycoside hydrolases reduced biofilm biomass by up to 94% [21–23].

Dispersin B is a glycosidic hydrolase that hydrolyzes PIA in order to disperse and eliminate EPS in medical devices [24]. As an important component of EPS, PIA is responsible for intercellular adhesion in *Staphylococcus epidermidis* (*S. epidermidis*). PIA production is catalyzed by various glucuronyltransferases (Gtfs) encoded by the icaADBC operon [25], which is negatively regulated by icaR, a transcriptional repressor of the icaADBC operon [26]. icaR repression of ica operon transcription contributes to a reduced production of PIA and impairs biofilm production [26]. Mu et al. [27] found that actinomycin D exhibited antibiofilm activity by reducing PIA production and cell surface hydrophobicity.

Glucosyltransferases (Gtfs) represent the primary virulence factors of *Streptococcus mutans* (*S. mutans*). These enzymes swiftly adhere to the salivary pellicle and hydrolyze dietary saccharose, forming glucans [28,29]. Consequently, they initiate the formation of extracellular polysaccharides that induce the formation of biofilm. It appears that glucans within the EPS could increase the preservation of protons as they approach the *S. mutans* cell surface, triggering the adaptive reaction to acid [30]. The acid tolerance response in *S. mutans* exhibits a distinct connection with the EPS matrix. This relationship is evidenced by the transcriptional regulation of the atp operon, responsible for encoding the F1F0-H/ATPase complex, in response to external pH changes in *S. mutans* [31]. In addition, Gtfs could synthesize EPS glucans that probably incorporate prospective targets such as GtfB, GtfC, and GtfD [32,33].

2.2. Extracellular proteins

EPS contains a large number of proteins, commonly including enzymes and structural proteins. During bacterial starvation, microorganisms secrete enzymes capable of degrading EPS components to generate energy; these enzymes can also act on the EPS of homogeneous bacteria or other species. Participating in the construction of the extracellular polysaccharide matrix are proteins within the EPS matrix, including both structural and non-

Table 1
Compositions and functions of extracellular polymeric substances (EPS) in biofilms.

Component	Bacteria	Location	Name	Function
Polysaccharides	*Bacillus subtilis*	Extracellular	epsA-epsO operon-encoded exopolysaccharide	Bacterial adhesion, EPS stability, permeability barrier
		Extracellular	poly-γ-glutamate (γ-PGA)	Bacterial adhesion, EPS scaffolding, nutrient source
	Candida albicans	Extracellular cell wall	α-Mannans	Forming mannan-glucan complex (MGCx), EPS scaffolding, inhibiting antibiotic penetration and host immunity
		Extracellular cell wall	β-Glucans	Forming mannan-glucan complex (MGCx), EPS scaffolding, inhibiting antibiotic penetration and host immunity
	Escherichia coli	Extracellular	Colanic acid	Biofilm formation, protection against harsh environments
	Haemophilus influenzae (nontypeable)	Extracellular	Lipooligosaccharide	–
	Pseudomonas aeruginosa	Extracellular	Alginate	Providing hydration and resistance to physical forces, and inhibiting antibiotic penetration and host immunity
		Extracellular/cell-associated	Psl	Providing structural integrity, inhibiting antibiotic penetration and host immunity
		Extracellular/cell-associated	Pel	Providing structural integrity, inhibiting antibiotic penetration
	Streptococcus mutans	Extracellular/cell-associated	Glucans/fructans	Biofilm formation, EPS scaffolding, nutrient source, cell-to-cell binding, protection against antibiotics
	Staphylococcus aureus	Extracellular	Polysaccharide intercellular adhesin (PIA) or poly-β(1-6)-N-acetylglucosamine (PNAG)	Bacterial adhesion and cohesion, EPS scaffolding, maintaining stability, protection against antibiotics
	Staphylococcus epidermidis	Extracellular	PNAG	Bacterial adhesion and cohesion, maintaining EPS stability
	Salmonella enterica serovar Typhi/ Typhimurium	Extracellular	Colanic acid, O-antigen capsule, Cellulose, Vi-antigen	Protection against immune response, bacterial adhesion
	Vibrio cholerae	Extracellular/cell-associated	Vibrio polysaccharide (VPS)	Bacterial adhesion and cohesion, maintaining EPS stability
Nucleic acids	Distribution in bacteria, archaea, and fungi	Extracellular	eDNA	Strengthening the biofilm matrix, protection against immune response, nutrient source during starvation
Proteins	*Bacillus subtilis*	Extracellular	Biofilm surface layer protein (BslA)	Surface hydrophobicity, permeability barrier
		Extracellular cell wall	Translocation-dependent antimicrobial spore component (TasA)/TasA anchoring and assembly protein (TapA)	EPS scaffolding, cell-to-cell binding
		Cell-associated	Flagellum	Bacterial adhesion and motility, mechanosensing
	Candida albicans	Cell wall	Agglutinin-like sequence protein (Als)	Bacterial adhesion, cell-to-cell binding, bacterial-fungal interaction
		Cell wall	Hyphal wall proteins (Hwp)	Bacterial adhesion, cell-to-cell binding
		Cell-associated/extracellular	Heat-shock proteins (Hsp70)	–
		Extracellular	Functionally classified enzymes	Metabolism of carbohydrate/amino acid/lipid/ nucleotide/energy/vitamins, translation, folding sorting, replication
	Haemophilus influenzae (nontypeable)	Extracellular	Type IV pilin protein	Enhancing the stability and strength of the matrix
	Pseudomonas aeruginosa	Cell-associated	Type IV pilins (T4P)	Bacterial adhesion, twitching motility, mechanosensing
		Cell-associated/extracellular	Lectins (LecA/LecB)	Bacterial adhesion, cell-to-cell binding
		Extracellular	CdrA	Bacterial adhesion and aggregation, biofilm matrix cross-linker
	Streptococcus mutans	Extracellular	Dextranase	EPS degradation/remodeling
		Cell-associated/extracellular	Glucosyltransferases (Gtf)/ fructosyltransferases (Ftf)	EPS formation
		Cell-associated	P1 (also referred to as Antigen I/ II)	Bacterial adhesion, cell-to-cell binding
		Cell-associated/extracellular	Glucan binding proteins (GbpA, GbpB, GbpC)	Bacterial adhesion and cohesion
	Staphylococcus aureus	Cell-associated/extracellular	Fibronectin-binding proteins (FnBPs)	Bacterial adhesion, engagement in homophilic interaction, biofilm formation
		Extracellular	Phenol soluble modulins (PSMs)	Proinflammatory, lysing of host cells, biofilm formation and spreading
		Cell-associated/extracellular	Staphylococcal protein A (SpA)	Bacterial adhesion, cell-to-cell binding, immune evasion
		Cell-associated/extracellular	*S. aureus* surface protein G (SasG)	Bacterial adhesion, cell-to-cell binding
		Extracellular	Biofilm associated protein (BAP)	Bacterial adhesion, cell-to-cell binding, biofilm formation

(*continued on next page*)

Table 1 (*continued*)

Component	Bacteria	Location	Name	Function
	Staphylococcus epidermidis	Extracellular	Phenol soluble modulins (PSMs)	Biofilm maturation
	Salmonella enterica serovar Typhi/ Typhimurium	Extracellular	Curli (amyloids), BapA, DNABII proteins (IHF and HU)	Biofilm maturation, enhancing the stability and strength of the matrix
	Vibrio cholerae	Cell-associated	Flagellum	Bacterial adhesion and motility
		Cell-associated	Mannose-sensitive hemagglutinin (MSHA) pili	Bacterial adhesion and motility
		Cell-associated/extracellular	Biofilm-associated protein (Bap1)	Bacterial adhesion, surface hydrophobicity, enhancing the stability and strength of the matrix, protection
		Cell-associated/extracellular	Rugosity and biofilm modulators (RbmA/RbmC)	RbmA: cell-to-cell binding RbmC: scaffolding, stability
Lipids	*Candida albicans*	Cell-wall/extracellular	Glycerolipids, sphingolipids	–
	Staphylococcus aureus	Cell-associated/extracellular	Teichoic and lipoteichoic acids	ECM formation, protection against antibiotics

enzymatic proteins. These proteins comprise cell surface-associated and extracellular carbohydrate-binding proteins, commonly referred to as lectins. Surface glycoproteins, membrane-associated glycoproteins, flagelli/pilli-associated glycoproteins, cellular glycoproteins, and secretory glycoproteins are distributed in a variety of ways in bacterial cells [34]. In addition, certain functional amyloid proteins (such as curli fibers), fimbriae, pili, and flagella are capable of forming nanotubes or nanofibers. These components promote cell adhesion to living and non-living surfaces and play important roles in the formation of the bacterial biofilm matrix and the transmission of pathogens [35]. These biofilm-related proteins could be potential targets to inhibit biofilm formation. Proteinase K, as a serine protease that degrades proteins by severing the C-terminal peptide bond, has been reported to suppress the proteinaceous adhesive force during the adhesion of EPS to a surface [36,37]. It is of vital importance for exoproteins to retain and decorate the EPS [38,39]. Certain proteins, such as DNA-binding proteins (DNABPs), biofilm-associated proteins (Baps), and other functional amyloid/amyloid-like proteins (FA/ALPs), are crucial participants in surface and EPS scaffolding adhesion as well as the monolithic physical stabilization of the biofilm matrix [40,41].

Bacteria are capable of producing functional amyloids, and there is typically one primary amyloid-forming protein, accompanied by other supplementary proteins that contribute to the production, secretion, or nucleation of fibrillization [42]. According to reports, the characteristics of amyloid promote the integrity and structure of biofilms [43]. Consequently, ascertaining amyloid-forming proteins and molecules and preventing their fibrillization represents a prospective approach for effective therapeutic intervention to combat biofilm [44].

Similarly, the utilization of enzymes to digest EPS exoproteins is probably the most promising strategy for inducing a massive biofilm dispersal phenomenon. In the gram-positive pathogen, there are 10 secreted proteases that have been identified to contribute to biofilm dispersal in *Staphylococcus aureus* (*S. aureus*). Aureolysin, two staphopains (SspB and ScpA), and V8 serine protease (SspA) have been revealed to participate in biofilm destruction [45–47]. It has been reported that purified aureolysin, ScpA, and SspB, promote the dispersal of established *S. aureus* biofilms [46]. In addition, LapG protease [48], Spl proteases [49,50], *Streptococcal* cysteine protease (SpeB) [51,52], surface-protein-releasing enzyme (SPRE) [53], trypsin [54,55], and so on, are able to degrade proteins.

2.3. Extracellular DNA (eDNA)

eDNA denotes the intracellular DNA released during cell lysis, exerting various effects. In numerous biofilms, eDNA functions as a structural scaffold within the EPS, enhancing bacterial adhesion and aggregation, and facilitating the horizontal transportation of genes [56,57]. The release of eDNA has been observed to occur independently, mediated by quorum sensing (QS), and is contingent upon the lysis of a specific subset of bacterial cells. It has been widely studied that the release of eDNA from gram-negative bacteria is mediated by both QS-dependent and QS-independent mechanisms [58–60]. The QS-independent mechanism predominantly governs the basal release of eDNA, initiating cell lysis through the regulation of flagella and type IV pili [61]. Whereas, the QS-dependent mechanism is primarily responsible for enhancing cell lysis and releasing a large quantity of eDNA. As for gram-positive bacteria, the release of eDNA is primarily induced by QS-dependent lysis of bacterial cells mediated by autolysins. The eDNA of biofilms is provided by the split of cell subsets. Jennings et al. [62] discovered that the mushroom-like microcolonies of *P. aeruginosa* carry a large amount of negative charge in their stalks, and eDNA can maintain the structural and functional stability of biofilms by interacting with positively charged polysaccharides (such as Pel) or other cations, thereby developing resistance to antibiotics. Researchers identified the cationic surfactant as a chelating agent that binds to eDNA [63]. This combination effect was similar to the enzymes on the matrix in EPS degradation [64]. All the EPS compositions and their functions are characterized in Fig. 1.

3. Current analytical approaches for the characterization of EPS composition

To investigate the structure and functionality of biofilms, a comprehensive analysis of EPS is a prerequisite. The purpose of EPS analysis is to elucidate the biofilm composition and biofilm adjustment to survival stress, including anti-biofilm agents and environmental changes. Consequently, accurate analysis of biofilm based on the EPS composition holds immense promise for the development of anti-biofilm agents. The analysis of EPS composition is highly dependent on analytical approaches and standard compounds used for quantification. The current analysis of EPS composition relies heavily on colorimetric approaches with a significant bias, which is likely due to the selection of a standard compound and the cross-interference of various EPS compounds in each assay. To counteract this, more sensitive spectrophotometric methods such as Raman microscopy (RM), surface-enhanced Raman scattering (SERS), Fourier transform infrared (FTIR) spectroscopy, were utilized to achieve accurate *in situ* analysis of biofilms. In addition, fluorescence labeling combined with CLSM enables an *in situ* and non-destructive chemical analysis of biofilm EPS. Table 2 provides a summary of the

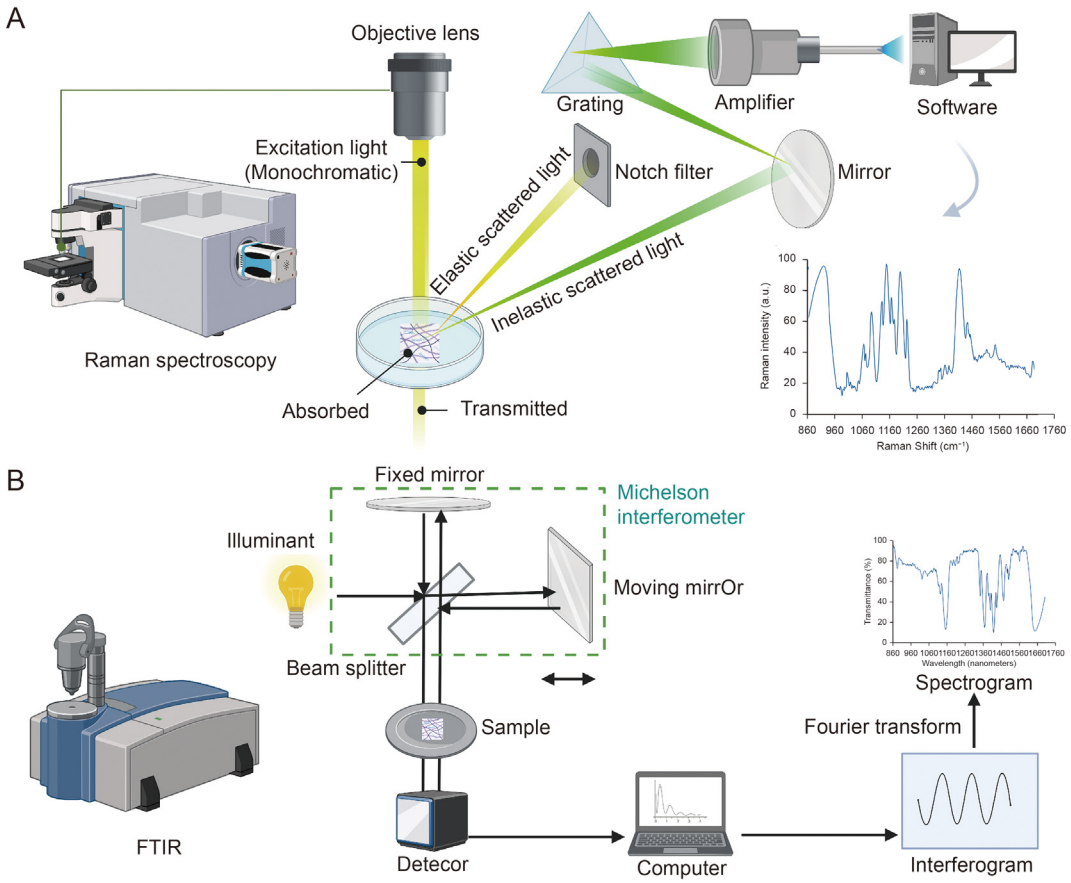

Fig. 1. The working principle of (A) Raman spectroscopy and (B) Fourier transform infrared (FTIR) spectroscopy.

characteristics, principles, advantages, disadvantages, and uses of these analytical approaches.

3.1. Colorimetric analytical assay

Most EPS fractions, comprising saccharides and proteins, are quantified using colorimetric methods, all of which are based on similar fundamental principles. Samples involving target compounds are used to react with a color reagent that results in an increase in absorbance intensity at a specific wavelength. Subsequently, the concentration of target compounds in the samples can be calculated by comparing the absorbance intensity values to those of a known concentration standard compound. In this regard, colorimetric methods could be used to detect the concentration of target compounds in EPS, such as proteins, saccharides, and uronic acids. Colorimetric methods are inexpensive, easy to manipulate, and time-saving [65,66]. Nevertheless, it should be noted that the corresponding equivalent of the analyzed compound in EPS varies according to the standard compound used, which determines the sensitivity of colorimetric methods based on the choice of standard. In addition to the standard, the applied methods influence the obtained equivalent in EPS. Using the same EPS sample and the same protein standard, the Lowry method and bicinchoninic acid (BCA) assay may produce different equivalents.

Generally, phenol-sulfate or anthrone-sulfuric assays are used to detect the content of extracellular polysaccharides [65]. Glucose is frequently used as the standard. The colorimetric methods for testing polysaccharides include the carbazole-sulfate method [67], the anthrone-sulfate method, the phenol-sulfate method [65], the interhydroxybiphenyl method, the 3,5-dimethylphenol method, and the 3,5-dinitrosalicylic acid (DNS) method, which measure polysaccharide content according to monosaccharides after the complete hydrolysis of polysaccharides under acidic conditions. In brief, polysaccharides are oxidized into furfural by concentrated sulfuric acid, which then reacts with the color reagent; thus, the absorption value in a certain wavelength range is directly proportional to the concentration.

In the determination of acidic polysaccharides in uronic acid, the sulfate-carbazole method, the interhydroxybiphenyl method, and the 3,5-dimethyl phenol method are widely used. While neutral polysaccharides interfere significantly with the carbazole-sulfate method, its specificity is relatively low, and its accuracy must be verified further. However, by adding a small amount of amino sulfonate, the interhydroxybiphenyl method can neutralize the influence of neutral sugars, and 3,5-dimethylphenol can eliminate the interference of neutral sugars using the dual-wavelength absorbometer algorithm. Typically, the total polysaccharide content is measured using the phenol-sulfate method or the anthrine-sulfate method. However, the color reagent of the anthrine-sulfate method is easily oxidized, resulting in low repeatability and a poor linear relationship. After the phenol-sulfate method is modified, the chromogen (phenol reagent) has higher stability, greater sensitivity, and less impurity interference, allowing for widespread application.

Using the phenol-sulfate or anthrone-sulfuric assay, a variety of potential anti-biofilm agents have been identified in the field of biofilm research, such as ferulic acid [68], vanillic acid [69], vanillin [70], thyme oil [71], essential oils [72], the extracts of *Bergenia*

Table 2
Current analytical approaches for EPS composition.

Approach	Component	Characteristic/principle	Advantages/disadvantages(A&D)	Qualitative/quantitative
Colorimetric assays	Polysaccharide	Sulfate-carbazole method Sulfate-anthrine method Sulfate-phenol method Interhydroxybiphenyl method 3,5-Dimethylphenol method 3,5-Dinitrosalicylic acid (DNS) method	A: cheap, easy to manipulate and less time-consuming; D: cross-interference and high dependence on the standard	Quantitative
	Protein	Lowry method BCA assay	A: cheap, easy to manipulate and less time-consuming; D: unreliable	Quantitative
	eDNA	Absorbance measurement at 260 nm Quantitative test	A: cheap, easy to manipulate and less time-consuming; D: Not found in current studies	Quantitative
Confocal microscopy	Polysaccharide	ConA targeting alpha-D-glucopyranose WGA targeting poly-N-acetyl-β-(1-6)-glucosamines (PIA/PNAG)	A: high specificity, microscopic in situ and non-destructive detection D: time-consuming staining procedures, unknown EPS components without specific staining reagents is impeded	Qualitative, quantitative
	Protein	SYPRO, Ruby or FITC		
	eDNA	40,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI)		
RM	Polysaccharide, protein, and DNA	The effect of an inelastic light scattering by molecules derived from vibrational interactions between laser radiation and molecular bonds	A: whole-organism fingerprints for biological samples, no sample preparation or sample pretreatment, non-destructive analysis, real-time monitoring, high-resolution spectra D: Weak signal, limited sensitivity and time-consuming, short-range, fluorescent interference, limited chemical information with a few bands	Qualitative, quantitative
SERS	Polysaccharide, protein, and DNA	The Raman effect can be substantially amplified when immediately approaching to a nanometer-roughened metal surface.	A: High sensitivity, non-destructive analysis, real-time monitoring D: Low signal to noise ratio, high requirement for the preparation of metal nanoparticles, matrix effect, limited research objects	Qualitative, quantitative
FTIR	Polysaccharide, protein, and DNA	Infrared radiation, the light emitted by the light source is converted into interferometer light.	A: High precision, wide range, non-destructive analysis of biofilms *in situ*, high-throughput D: Expensive, high requirements for sample preparation, signal interference	Qualitative, quantitative
HPAEC-PAD	Polysaccharide	The ability of sugars to dissociate into anions in a high pH (>12) medium	A: efficient, rapid, and trace (pmol ~ nmol) separation and detection D: relying on instruments with many steps	Qualitative, quantitative

Note: eDNA: extracellular DNA; BCA: bicinchoninic acid; ConA: concanavalin A; WGA: wheat germ agglutinin; PIA/PNAG: polysaccharide intercellular adhesin/poly-N-acetyl-β-(1-6)-glucosamines; EPS: extracellular polymeric substances; FITC: fluorescein isothiocyanate; RM: Raman microscopy; SERS: surface-enhanced Raman scattering; FTIR: Fourier transform infrared; HPAEC-PAD: high-performance anion exchange chromatography-pulsed amperometric detector.

crassifolia leaves [73], paeoniflorin [74], ginger extract [75], the extract of *Actinidia deliciosa* [76], piper betle ethyl acetate extract [77], andrograpanin [78], D-amino acids (D-AAs) [79,80], sulfonated chitosan (SCS) and chitosan hydrochloride (WCS) [81], and chitosan N-acetylglucosaminohydrolase [82]. Concurrently, the majority of anti-biofilm agents are used to quantify the extracellular protein content with the BCA protein assay, Bradford assay, or Lowery assay using bovine serum albumin as the standard. For eDNA quantification, the eDNA content in samples is generally extracted with a bacterial DNA extraction kit, and the absorbance at 260 nm is measured using a ultraviolet-visible (UV-Vis) spectrophotometer.

3.2. Fluorescence labeling combined with CLSM

In biofilms, bacterial cells densely and intensively aggregate, which is considered a typical characteristic of biofilm formation. Scanning electron microscopy (SEM) is commonly employed to visualize these aggregation states. However, EPS forms gel-like structures; therefore, direct observation of bacterial cell microstructures through SEM does not provide a comprehensive understanding of EPS composition. Specialized characterization approaches are necessary to analyze the properties of EPS.

CLSM is a standard technique utilized for 3D visualization of biofilm architecture. With specific fluorescence labeling, CLSM could better reflect the 3D architecture changes corresponding to the EPS contents of biofilms, enabling quantitative assessment of EPS distribution within the biofilm matrix. Fluorescence dyes are typically used to image EPS components, including fluorescein isothiocyanate (FITC)-labeled type IV concanavalin A (ConA) and wheat germ agglutinin (WGA) for polysaccharides [70,75,83–86], SYPRO Ruby [70,75] or FITC [86] for proteins, and 4',6-diamidino-2-phenylindole (DAPI), or propidium iodide (PI) for eDNA [70,75].

In general, these dyes have specific labeling principles. For instance, lectins are a series of proteins or glycoproteins derived from plants, animals, or microorganisms that have a special affinity for carbohydrates [87,88]. In this regard, fluorescence-labeled lectins are designed as probes to observe different glycoconjugates in EPS, enabling microscopic, *in situ* detection of the distribution of EPS and its components in biofilms [89–91]. Intriguingly, different lectins can exhibit specific recognition properties for a specific sugar monomer. ConA, the lectin of *Canavalia ensiformis*, can recognize glucose and mannose residues and is a well-studied example. Consistently, WGA targets poly-N-acetyl-β-(1-6)-glucosamines (PIA/PNAG), which contribute to bacterial aggregation.

In conclusion, the combination of fluorescence labeling and CLSM enables a specific *in-situ* analysis of the composition of EPS and their distribution. Nevertheless, this technique, which is highly dependent on specific reagents, requires time-consuming staining procedures and has limited specificity, as the staining of unknown EPS components is impeded in the absence of specific staining reagents.

3.3. Conventional spectrophotometric methods

3.3.1. Raman spectroscopy (RM) and surface-enhanced Raman scattering (SERS)

In addition to CLSM analysis, combining the application of spectrometric techniques could provide valuable new insights into the *in situ* analysis of biofilms. RM is a prospective addition to the CLSM analysis in this regard. Emerging evidence indicates that the concurrent use of CLSM and RM can provide significant and novel insights into the composition and structure of the biofilm matrix.

RM is a technique founded on the Raman scattering phenomenon, originally discovered by C.V. Raman, which analyzes the scattering spectrum to glean information about molecular vibration and rotation. It is widely employed in the study of molecular structure. When a transparent sample is illuminated with monochromatic light, the majority of the light passes through, while a small amount is scattered in all directions. Light scattering can be classified into Rayleigh scattering and Raman scattering [92]. Rayleigh scattering occurs when an elastic collision happens between photons and sample molecules, where no energy exchange transpires between the photon and the molecules. In this process, the energy of scattered light remains the same as that of the incident light, although the direction can be altered [93]. In contrast to Rayleigh scattering, Raman scattering occurs due to inelastic collisions between photons and sample molecules, leading to a difference in energy between the scattered light and the incident light. This phenomenon results in changes in both frequency and direction of light. The intensity of the scattered light is about 10^6–10^9 of the total scattered light intensity [94]. The reason for Raman scattering is the energy exchange between photons and molecules, which changes the energy of photons. This is the theoretical basis on which Raman spectroscopy can be used as an analytical tool for molecular structures [95]. Fig. 1A depicts the working principle of Raman spectroscopy.

RM is a non-destructive approach and can be applied to liquid, solid, and gaseous samples with minimal sample preparation and no sample staining. In addition, this technique achieves whole-organism fingerprints for biological samples with real-time monitoring and high spatial resolution. RM has been utilized for quantitative assessment and spatial distribution of biofilm matrix [96]. Moreover, RM has recently shown promise for the *in situ* chemical characterization of microbial EPS compositions [97]. However, RM suffers from a weak signal, limited sensitivity, and fluorescence interference, subsequently resulting in a time-consuming process. Moreover, Raman spectra typically consist of only a few bands and provide limited chemically informative evidence [98].

Intriguingly, the Raman effect can be enhanced significantly. SERS is the process by which the Raman effect is significantly amplified upon immediate proximity to a nanometer-roughened metal surface. Enhancement factors in the range of 10^3–10^4 can be achieved through electromagnetic and chemical enhancement. In special environments, such as closely spaced particles or rough nanostructures serving as “hot spots,” enhancement factors can reach 10^{15}, which is sufficient for detecting a single molecule [99,100]. This technique has been utilized for *in situ* characterization of multispecies biofilm matrix without damaging the sample, and recent evidence demonstrates that the enhanced sensitivity of SERS enables rapid biofilm analysis. The initial case involved the utilization of hydroxylamine hydrochloride-reduced silver colloidal nanoparticles (NPs) as the SERS substrate, which allowed for the uniform dispersion of metal NPs in the biofilm matrix. This technique produces SERS spectra that are reproducible and enhanced by several orders of magnitude [101].

Notably, due to the existence of the surface enhancement effect, the SERS signal is affected by numerous factors (such as the shape and size of NPs, molecular adsorption mode, etc.), resulting in a low signal-to-noise ratio. In addition, SERS technology requires the preparation of high-quality and uniform metal NPs, and strict control of experimental parameters. Moreover, SERS technology requires samples to be adsorbed on the surface of NPs, hence it is primarily used in the analysis of small molecules in chemical and biological fields.

3.3.2. Fourier transform infrared (FTIR) spectroscopy

Infrared spectroscopy (IR) is primarily an analytical approach used to determine the molecular structure of substances and identify compounds based on the relative vibration and rotation of the atoms within the molecule. In the analysis of molecular structure and chemical composition, IR serves as a valuable tool, often referred to as the “fingerprints of molecules” [102,103].

The infrared absorption spectrum is formed by molecular vibrations and rotational transitions. The atoms forming chemical bonds or functional groups are in a state of constant vibration (or rotation), and their vibration frequency is equal to that of infrared light. Therefore, when molecules are irradiated with infrared light, chemical bonds or functional groups within the molecules can absorb vibrations. Different chemical bonds or functional groups have different absorption frequencies and may occupy various positions in the infrared spectrum, providing crucial information to confirm the type of chemical bonds or functional groups present in the molecules [104].

The FTIR instrument consists of a light source, a Michelson interferometer, a sample pool, a detector, and a computer. The light emitted by the light source is converted into interferometer light, which contains information about all its wavelengths. When the interference light passes through the sample, some wavelength of light is absorbed by the sample and becomes the interference light containing the sample information. The sample interference map is collected by the computer, and the absorbance or transmittance of the infrared spectrum changes with frequency or wavelength [105,106]. Fig. 1B depicts the working principle of FTIR spectroscopy.

As a less conventional method in pathogen research, FTIR offers a rapid and nondestructive approach applicable to various biological systems. Recent studies have verified the viability of FTIR for non-destructive, *in situ* analysis of biofilms. Each absorption spectrum reveals informative details about the molecular structure of the sample, predominantly concerning the functional groups derived from carbohydrates, proteins, and DNA. It demonstrates powerful qualitative function when combined with chromatography (gas chromatography (GC)-IR or thermal gravimetric analysis (TGA)-IR). Examples include *in situ* characterization of biofilm formation using a combined microscopic and spectroscopic approach involving FTIR and gas chromatography-mass spectrometry (GC-MS) [107].

In conclusion, FTIR can provide numerous benefits for the *in situ* characterization of biofilm formation, including: (1) High precision: FTIR technology can provide very high resolution, enabling it to detect minute changes in samples and rapidly analyze large amounts of data; (2) Wide range: FTIR can analyze over a wide range of wavelengths, from visible to far-infrared light, enabling it to detect different regions of numerous samples; (3) Non-destructive: Compared with other spectroscopic technologies, FTIR is non-destructive, can analyze samples in solid, liquid, and gaseous states, and requires no special treatment; (4) High-throughput: FTIR technology can rapidly scan samples to analyze large amounts of data in a brief period of time. However, it is also important to realize that FTIR is an advanced, high-priced analytical instrument with high requirements for sample preparation (high sample transparency and optical properties) and that the FTIR

signal is susceptible to interference from impurities such as water vapor and CO_2, which can affect the accuracy of experimental results.

3.3.3. Analysis of monosaccharides in EPS by a high-performance anion exchange chromatography-pulsed amperometric detector (HPAEC-PAD)

Exploiting the capacity of sugars to dissociate into anions in high-pH (>12) conditions allows for ion exchange chromatography on a compacted thin-shell anion exchange resin column. The individual sugar constituents are subsequently identified using PAD, enabling efficient, swift, and sensitive separation and detection in the picomole to nanomole range.

HPAEC-PAD analysis is conducted to identify the existence of monosaccharides and unravel the intricate diversity of sugars within the polymers of EPS. In the initial elution with diluted sodium hydroxide, the presence of one sugar alcohol, two amino sugars (glucosamine), and seven neutral sugars was verified. Subsequent elution with sodium acetate and sodium hydroxide revealed the presence of galacturonic acid and glucuronic acid in EPS [108].

3.3.4. High-specificity methods of monosaccharides in EPS

P. aeruginosa contains three extracellular polysaccharides, including alginate, Pel, and Psl. Alginate interacts with divalent cations to form dense hydrogels, which contribute to PA biofilm formation and architecture. Meanwhile, alginate provides a survival advantage by protecting bacterial cells from phagocytosis. An important consequence of alginate overexpression lies in the formation of mucoid *P. aeruginosa*, which increases antibiotic resistance. Similar to general polysaccharide quantification, alginate quantification utilized colorimetric approaches using 1% phenylphenol solution [109] or cetylpyridinium chloride [83] as the color reagents. In contrast to the role of alginate in mucoid *P. aeruginosa*, Psl is required for biofilm development in non-mucoid strains as the primary biofilm polysaccharide. Another study demonstrated that a high-specificity strategy can be used to quantify Psl content. Generation of Psl antiserum was performed in New Zealand white rabbits immunized with the crude Psl material. Subsequently, using the obtained Psl antiserum, Psl content was quantified by Western immunoblotting and enzyme-linked immunosorbent assay (ELISA) [109]. This method relied heavily on specific antiserum and required constant antibody preparation, which appeared to be a time-consuming and laborious procedure. Fig. 2 lists all the analytical approaches mentioned.

3.4. Challenges and proposed research plan for the characterization of EPS compositions

Interpretations based on the EPS components can only be performed accurately if the values are reasonably precise. Previous research has demonstrated that colorimetric analytic methods for saccharide quantification and protein analysis have certain inaccuracies. Interfering substances and measurement techniques can readily result in an underestimation or overestimation of the actual content [108]. Fig. 3 shows the possible cross-interferences of components present in EPS with colorimetric methods.

Recognizing the pivotal role of EPS in biofilm functioning, it is imperative for EPS research to delve into a profound analysis of its intricate compositions, moving beyond the current reliance on colorimetric analytic methods. This transition necessitates an exhaustive examination of EPS constituents, promising to elevate biofilm research to an unprecedented level of understanding.

In current EPS research, the anthrone sulfuric acid method and the phenol sulfuric acid method, which were discovered in the 1950s and have been used for over 70 years, are still extensively utilized. The colorimetric methods employed for biofilm EPS analysis exhibit significant drawbacks, including low repeatability, comparability, and specificity. Their efficiency and cost-effectiveness are compromised. While the utilization of correction factors or appropriate standards can enhance these approaches, it is unlikely to completely mitigate all the limitations. Therefore, colorimetric analytic methods should only be used to illustrate general information about EPS composition. The EPS research field necessitates a comprehensive analysis of diverse compositions and a meticulous interpretation of compounds. The high heterogeneity and complexity of carbohydrates within the polymers, encompassing functions and precise sugar bonding, demand further elucidation and scrutiny. The versatile analytical strategies and methods, particularly spectrophotometric analysis, used in the study of plant-derived polysaccharide research could be substantially helpful in the study of polysaccharides in EPS, although many of these have not yet been extensively applied to the EPS field. The molecular weight distribution, monosaccharide composition and its molar ratio, and glycosidic bond analysis are proposed as methods for polysaccharide identification in order to achieve this aim.

3.4.1. Proposed research scheme for polysaccharide characterization

3.4.1.1. Determination of the relative molecular weight distribution. Polysaccharide is a macromolecular polymer composed of more than 10 different or identical types of monosaccharides, which makes the molecular weight of polysaccharides heterogeneous and polydisperse. Pure polysaccharides consistently span a wide range of molecular weights. The viscosity method, osmolality method, ultracentrifugation method, end-base method, and light dispersion method are frequently employed to determine the relative molecular weight of polysaccharides. Chromatography such as gel chromatography (GPC) and high-efficiency gel permeation chromatography (HPGPC) are introduced subsequently [110,111].

3.4.1.2. Monosaccharide composition and its molar ratio. Monosaccharides are the basic constituents of polysaccharides and contain chiral polyhydroxyaldehyde or polyhydroxyketone structures. Glucose, mannose, rhamnose, and arabinose are the most common monosaccharides. Currently, the determination of monosaccharides and their molar ratios is an effective method to identify polysaccharides. Polysaccharides are macromolecular polymers, composed of high-polarity monosaccharides, which are non-volatile and without an ultraviolet absorption group, so the pretreatment of hydrolysis derivation is required prior to the analysis of the polysaccharide, followed by testing the monosaccharide composition using thin chromatography (TLC), GC, high-performance liquid chromatography (HPLC), high-performance capillary electrophoresis (HPCE), fluorescence-assisted sugar electrophoresis (FACE), and so on. The commonly used derivative reagents are trimethylsilanther, 1-phenyl-3-methyl-5-pyrazolinone (PMP), 8-aminobanaphthalene-1,3,6-trisfonate (ANTS), sugar alcohol acetate, N, O-Bis(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (BSTFA + TMCS, 99:1, *V/V*), trimethylsilyl oxime, methyl ether, and 1-aminopyrene-3,6,8-triisfonic acid (APTS), which can effectively improve the volatility or UV absorption of polysaccharides. HPLC and GC analyses are most widely used.

3.4.1.3. Glycosidic bond analysis. The glycosidic bond of polysaccharides refers to the bond formed between the same or different monosaccharides after dehydration and condensation, which is primarily determined by chemical methods and

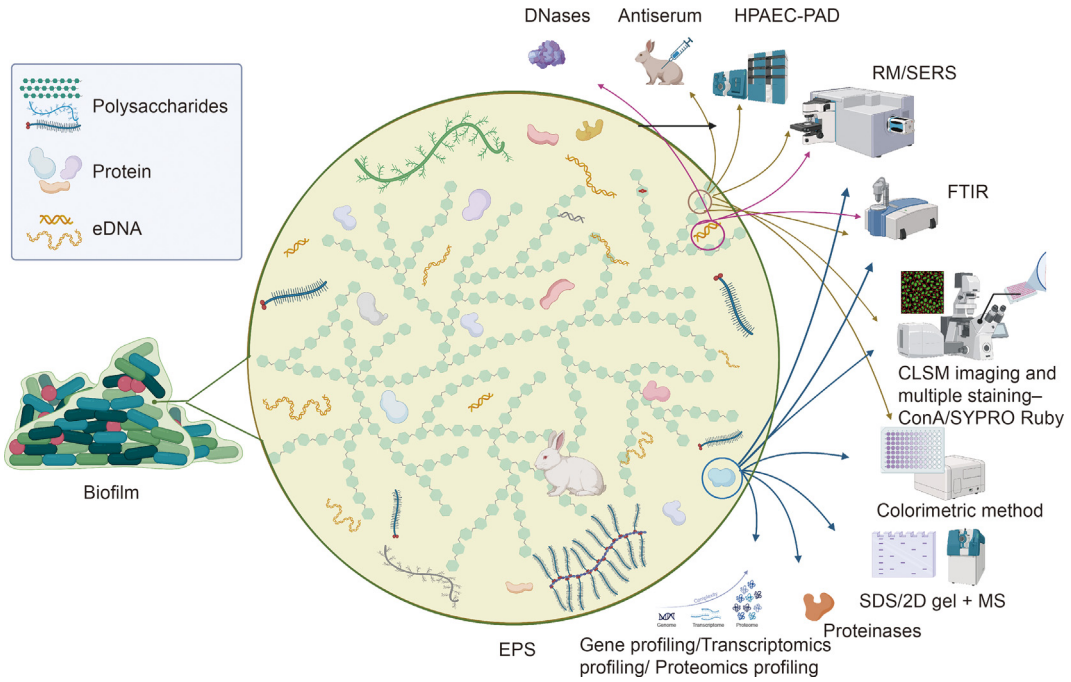

Fig. 2. Current analytical approaches for extracellular polymeric substances (EPS) composition. eDNA: extracellular DNA; HPAEC-PAD: high-performance anion exchange chromatography-pulsed amperometric detector; RM: Raman microscopy; SERS: surface-enhanced Raman scattering; FTIR: Fourier transform infrared spectroscopy; CLSM: confocal laser scanning microscopy; ConA: concanavalin A; SDS: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2D gel: two-dimensional gel electrophoresis; MS: mass spectrometry.

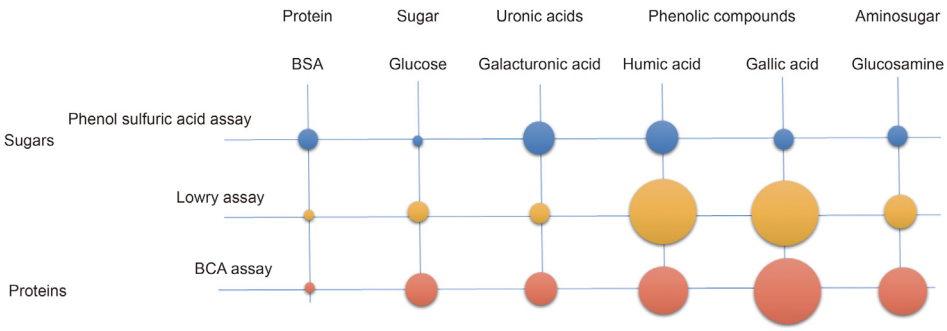

Fig. 3. Possible cross-interferences of components present in extracellular polymeric substances (EPS) with colorimetric methods. BSA: bovine serum albumin; BCA: bicinchoninic acid.

instrumental analysis. Polysaccharides are easily oxidized as they contain aldehyde (−CHO), ketone (−C═O), primary alcohol ($R-CH_2-OH$), secondary alcohol (R1−CH−(R2-)−OH), and o-diol group structural units [112]. With the advancement in sample pretreatment technology, methylation reactions have come to the fore [113]. In acidic conditions, the unbound hydroxyl groups in polysaccharides are replaced by methylation reagents, selectively cleaving glycosidic bonds. Subsequently, the sites resulting from bond cleavage in polysaccharides are linked to corresponding derivative reagents. Spectrophotometric analysis can then be employed to determine monosaccharide composition and the binding sites of glycosidic bonds. The primary instrumental-analysis methods include nuclear magnetic resonance spectroscopy (NMR), IR, mass spectrometry (MS), GC-MS, etc [114,115]. There are some characteristic absorption peaks in the polysaccharide structure. IR can identify information regarding the type of glycosidic bond, glycoring type, and glycosugar configuration based on the characteristic absorption peaks of hydroxyl groups; GC-MS can obtain a variety of structural information about the type of monosaccharide residue and the connection site of the glycosidic bond; and NMR can analyze the type of glycoside bond configuration and determine the number of sugar residues and some specific groups [116,117]. In addition, several new spectrophotometric instruments, such as X-ray diffraction (XRD) [118], atomic force microscopy (AFM) [119], and circular dichroism (CD) [120], play increasingly significant roles in glycosidic bond analysis.

This previously mentioned analytical methodology could analyze a specific polysaccharide of interest in EPS while minimizing interference from other polysaccharides. To ensure the precision and high efficiency of subsequent polysaccharide analysis in EPS, pretreatments such as the elimination of plant-derived polysaccharides are essential. For instance, plant-derived natural

products play an increasingly important role in the discovery of anti-biofilm agents to combat microbes [121]. However, the extracts of plant-derived natural products, such as herbal medicines, typically contain a substantial amount of polysaccharides. The interference from exogenous polysaccharides in plants appeared to be the obvious problem when endogenous polysaccharides in biofilm EPS were analyzed. Therefore, it is essential to consider removing plant polysaccharides from the plant extract prior to evaluating their antibiofilm effect in biofilms. Removing plant polysaccharides may be achieved by two methods: (1) preparing the serum collected after oral administration of plant extracts in animals, as polysaccharides are macromolecules that cannot be absorbed into the bloodstream; and (2) sugar fermentation by pectinase and amylase in plant extracts to minimize the plant-derived polysaccharides to the greatest extent. Fig. 4 is a flowchart for the proposed research scheme of polysaccharide identification and removal of plant polysaccharide.

3.4.2. Approaches for the characterization of other EPS compositions

In addition to polysaccharides, biochemical techniques and omics-based biofilms have been utilized for the identification of other EPS components. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis (2D-gel) can provide information about the sizes and varieties of proteins of interest, followed by mass spectrometry analysis that can reveal the features and functions of such proteins. In this regard, additional systemic omics-based techniques must be developed and implemented in the EPS system. Gene profiling, transcriptomics profiling, and proteomics profiling combined with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and quantitative real-time PCR are extensively used to evaluate the level of biofilm-related genes responsible for the synthesis and transport of EPS. Specifically, some commercial eDNA quantification kits use calf thymus DNA as the standard, including the Quant-iTTM ds BR assay kit [122] or PicoGreen dsDNA Quantification [80]. The eDNA content was subsequently characterized by the fluorescence intensity recorded by a microplate reader.

4. Novel approaches and strategies for promoting biofilm penetration through EPS

The poor permeability and low accumulation of therapeutic agents in the biofilm, which result from the special properties of biofilm matrix barriers, such as highly viscous compact EPS structures and negatively charged biofilm components, are the leading causes of the failure of therapy for biofilm-related infections. Due to the existence of EPS as a matrix barrier, current antimicrobial compounds are unlikely to reach the bacteria in the inner layers of mature biofilm [123]. Therefore, the enhancement of penetration into the biofilm is of great significance for antibacterial therapies targeting bacterial biofilm.

Enhancing antibiotic penetration and minimizing the interference of EPS are the most important factors in overcoming the biofilm matrix barrier. EPS targeting is a promising but underexploited strategy for combating biofilm, as it allows matrix disruption and promotes the penetration of antimicrobials to increase the susceptibility to antimicrobials. Traditional analytical approaches and pharmacological treatments targeting EPS seem inadequate to tackle this issue; therefore, a constant commitment to develop novel strategies with enhanced biofilm penetrability as promising alternatives to traditional approaches is essential.

In addition to utilizing bioactive enzymes to degrade EPS constituents to alter the integrity of EPS and promote drug penetration, versatile technologies, and biomaterials such as nanotechnologies, composite microneedles, clustered regularly interspaced short palindromic repeats (CRISPR) arrays, and phage therapy, play crucial roles in promoting the penetrability of therapeutic agents against the biofilm matrix.

4.1. Novel nanomaterials and biomaterials

4.1.1. Nanotechnology-based strategies

The application of nanotechnology in addressing pathogenic biofilm matrices represents a swiftly advancing approach. Over the past decade, mounting evidence has illustrated the effectiveness of nanotechnology-based strategies in combating pathogenic microorganisms. This is primarily attributed to the versatile utilization of nano systems as inherent antimicrobial agents.

The size of nanotherapeutic agents presents a significant impact on antibiofilm effects. The strengthened penetration and retention effect (EPR) in pathogenic microorganisms may allow nanotherapeutic agents to accumulate in biofilm [124]. In general, the biofilm can absorb NPs of about 300 nm in size. Nevertheless, the bacterial cell wall serves as a barrier, hindering the direct internalization of nanomaterials through endocytosis by bacteria [125]. However, nanomaterials with sizes below a certain threshold, such as < 50 nm, are believed to have the potential to penetrate bacterial cells. Metal NPs, in particular, exhibit extremely small dimensions and strong interactions with bacteria, with their antibacterial effectiveness primarily contingent upon their size. Several examples of NP-based antibiofilm strategies share common principles: plant extracts, antibiotics, or materials with antibacterial properties are used to conjugate metal NPs (Ag, Cu, Au, etc.), metal oxide NPs (ZnO, Fe_2O_3, CuO, etc.), surfactants, and polymers to harvest composite NPs. These newly-formed nanomaterials could overcome the matrix barrier by penetrating the pores and spaces of the EPS and then reaching the bacterial cells embedded in pathogenic biofilms to sufficiently exert their antibacterial actions. Fig. 5A displays these EPS targeting strategies using NPs.

A plant extract from *Heliotropium crispum* (HC) was applied to Ag NPs to form stable HC-Ag NPs with an average particle size of 42 nm. These NPs showed excellent antibiofilm properties by disrupting biofilm EPS and bacterial cell walls. Cao et al. [126] synthesized a chitosan NP named 2-methacryloyloxyethyl phosphorylcholine-nanocapsule (PMPC-CS). It was discovered that PMPC-based surfaces exhibited less attachment of EPS, which strengthened the perforation of PMPC-CS and was advantageous for the accumulation and transmission of antimicrobials throughout the biofilm. Altaf et al. [127] discovered cerium oxide NPs (CeO_2-NPs) that were synthesized using Acorus calamus aqueous extract. The existence of CeO_2-NPs showed an antibiofilm effect by inhibiting up to 50% of EPS secretion in *S. aureus*, *P. aeruginosa*, and *E. coli* in a dose-dependent manner. In other words, EPS formation inhibition is one of the potential mechanisms of the anti-biofilm effect of CeO_2-NPs.

Ali et al. [128] subsequently synthesized biogenic hematite (α-Fe_2O_3) NPs of average size < 10 nm using a green approach with aloe vera extract (ALE) named ALE-α-Fe_2O_3NPs. Researchers evaluated the defensive effect of EPS to combat the antibiofilm activities of ALE-α-Fe_2O_3NPs in normal EPS producers (pristine) and experimentally modified (low-EPS) *P. aeruginosa* cells. The results revealed that ALE-α-Fe_2O_3NPs-bacterial interaction promoted the adhesion of NPs to the EPS surface, enhanced the movement of NPs within the EPS, and subsequently promoted the permeation into cells which ultimately led to the inhibition of biofilm growth by intracellular reactive oxygen species (ROS) activity.

Intriguingly, researchers reported dextran-coated iron oxide nanozymes (Dex-NZM) that demonstrated effective catalyzed (peroxide-like enzymes) strength in acidic conditions [129].

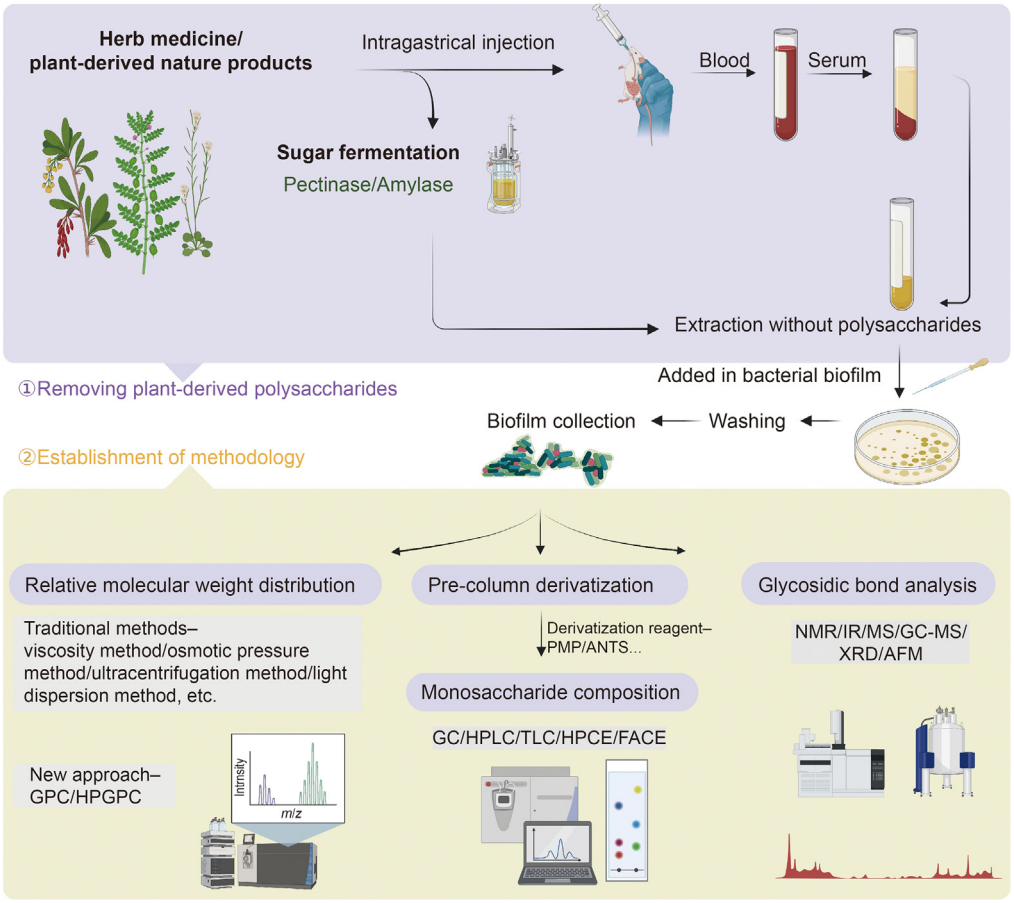

Fig. 4. A flow chat of proposed research scheme of polysaccharide identification.

Peroxidase-like substances can lead to the generation of free radicals that can eradicate bacterial cells of *S. mutans* and reduce EPS levels. Dextran-coating enhanced NZM affinity for the EPS structure, which stimulated hydrogen peroxide (H_2O_2) to promote sterilization and EPS-matrix disintegration. It was important to note that the dextran coating possessed high specificity for targeting biofilms. The results showed that Dex-NZM were incorporated into EPS to a greater extent than uncoated NZM, probably because their chemical properties were similar to dextran, which can be added into the EPS structure during glucan synthesis by *S. mutans*-derived GtfB exoenzymes through receptor responses. Meanwhile, the penetration depth of Dex-NZM into the inner biofilm was up to 40 μm, and its distribution was particularly uniform.

Besides, accumulating evidence has verified the potential of novel materials in anti-biofilm agents by inhibiting EPS production, including NPs co-loaded with farnesol and myricetin [130,131], biosynthesized baicalein-coated gold NPs [132], a natural curcuminoid-intercalated double-deck hydroxide nanohybrid, and mixed lipids of rhamnolipid [133] and phospholipids as the outer layer of NPs [134].

As previously mentioned, although the antimicrobial properties of NPs have been established and verified in biofilm control, the interaction mode between NPs and the biofilms remains unclear, as does the potential of NPs to induce EPS chemical and physical variability and heterogeneity. A comprehensive understanding of the interaction between NP and EPS could encourage researchers to develop more effective EPS-targeting strategies for biofilm control.

4.1.2. Composite microneedles (MNs)

MN patches include a variety of small and specific needles ranging in length from 0.1 to 1 mm on the supporting surface. These MNs were able to perforate the epidermis and dermis tissues without causing significant tissue injury and pain, as well as an obvious unfavorable inflammatory reaction. These MNs have been extensively used for subcutaneous drug delivery and have great potential for application in drug delivery [135]. Intriguingly, growing evidence suggests that MN patches can penetrate the cutaneous barrier and the bacterial biofilm barrier formed by EPS to realize efficient drug delivery within inner biofilms [136]. Fig. 5B illustrates these EPS targeting strategies using MNs.

Composite MNs of chitosan and zinc nitrate (CS—Zn [II] MNs) were also investigated for the removal of the bacterial biofilm. Due to their aciform structure, MNs were able to impale the EPS and efficiently transfer the CS and Zn^{2+} into the inner bacterial biofilm, while their large specific surface area accelerated the spread of antimicrobials in the bacterial biofilm [137]. Tian et al. [138] synthesized the zwitterionic mixed shell polymeric micelles (ZW-MSPMs). ZW-MSPMs became positively charged in the acidic condition of *staphylococcal* biofilms due to the inversion of the zwitterionic poly (quaternary-amino-ester) to a cationic lactone ring. Both *in vitro* and *in vivo*, ZW-MSPMs interacted intensively with important EPS elements, such as eDNA and proteins, to

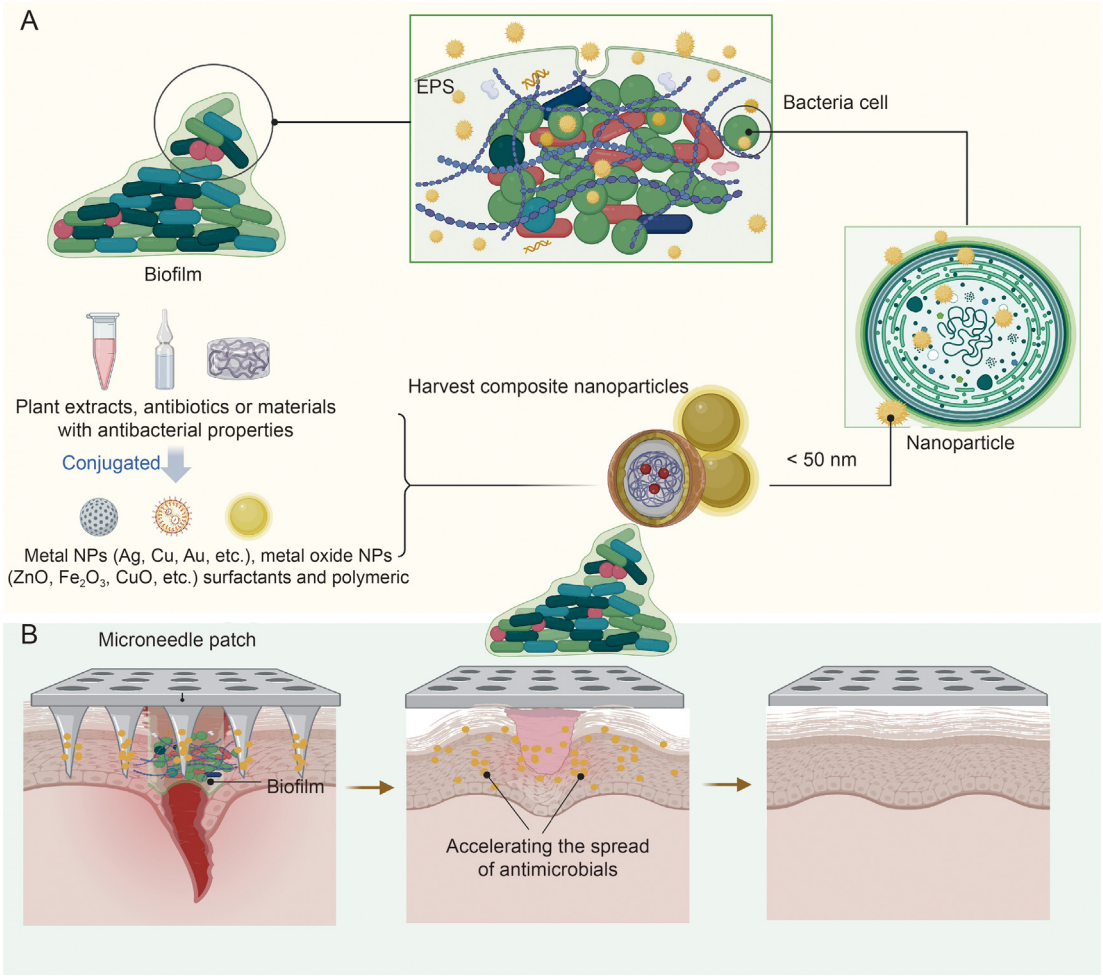

Fig. 5. Extracellular polymeric substances (EPS) targeting strategies using (A) nanoparticles (NPs) and (B) microneedles (MNs).

enhance antimicrobial distribution and, consequently, biofilm efficacy.

In addition, chemical bonds can be subtly incorporated into MN patches to achieve the rapid and targeted release of antimicrobial agents in biofilm-infected areas by cleaving these bonds in response to the biofilm microenvironment. A self-dissolving polyvinylpyrrolidone-based microneedle and a biodegradable polyvinyl acetate-based microneedle patch platform were developed to deliver antimicrobial agents (e.g., chloramphenicol, levofloxacin) and α-amylase (an exopolysaccharide hydrolase) into deep biofilm and trigger *in situ* release of these agents to maximize their antibacterial effects. The combined therapy with MNs eradicated the mature biofilm and inactivated the pathogen within the biofilm, indicating the promising potential of MN patches against biofilm infection [139,140].

4.1.3. Liposomes

Liposomes can fuse with phospholipid membranes and achieve facile penetration, thereby delivering antimicrobial agents directly to bacterial cells and maximizing therapeutic efficacy with fewer side effects [67]. Currently, no marketable liposome products are reported for the treatment of biofilm infections, while several liposomal formulations are in Phase II/III clinical trials, including Arikace (Transave, Inc.) [71], a formulation containing the antibiotic amikacin, and Fluidsomes [67,72], a formulation containing tobramycin, for the treatment of respiratory infections. Researchers specifically designed an EPS-targeting liposomal formulation loaded with DNase I and proteinase K and evaluated the anti-biofilm effect of the cationic liposomes in mature *Cutibacterium acnes* (*C. acnes*) biofilms. DNase I and proteinase K can target and decompose the biofilm degradation of EPS. The plausible mechanism of cationic liposomes loaded with DNase I and proteinase K was that the liposomes exhibited superior capability in degrading biofilm structures and the characteristic of facile penetration, which enabled DNase I and proteinase K to play more efficient roles in eDNA and protein degradation [141].

4.1.4. Other therapeutic biomaterials

In addition to the aforementioned strategies, new therapeutic biomaterials have also demonstrated great clinical efficacy. The first example comes from chemical oxidation in photocatalysis. It prefers to accelerate the transfer of complete biofilm rather than decelerate the spread of biofilm, which contributes to the destruction of the integral and unitive structure of biofilms. EPS, which sustains biofilm formation and survival, was disintegrated and eliminated after photocatalysis [137]. Researchers incorporated carbon-doped g-C3N4 into a chitosan matrix to form a photocatalytic and self-cleaning composite. During photocatalysis, ROS were observed to induce oxidation, decomposition, or removal of EPS in *S. epidermidis* and *P. aeruginosa* PAO1 biofilms. In addition,

there is more agglomeration of PIA within mature *S. epidermidis* biofilms formed on the composite surfaces in the dark, whereas after photocatalysis, there are only small clusters of PIA on the composite surfaces in biofilms. Notably, *E. coli* O157:H7 biofilms exhibit strong resistance to photocatalytic oxidation, which weakens the anti-biofilm capability of this composite. Different responses of EPS during photocatalysis may explain the discrepancy in biofilm eradicative efficacy between various bacterial strains, and this highlights the significance of EPS destruction for biofilm removal [61].

Xu et al. [142] discovered polyurethane urea (PUU) films without air plasma treatment (hydrophobic surfaces). The uneven surfaces with submicron-size patterns (400/400 nm and 500/500 nm) exhibited significantly less adhesion to the EPS than the smooth surfaces, indicating that the PUU was conducive to reducing the adhesion and accumulation of bacterial EPS. Hwang et al. [143] discovered a non-leachable antibacterial composite which had potent anti-biofilm ability when directly combined with polymerizable imidazolium-containing resin (anti-bacterial resin with carbonate linkage (ABR-C)) and converted into a methacrylate-based scaffold (ABR modified composite (ABR-MC)). Low-level incorporation of the imidazolium moiety (~2% (*m*/*m*)) ensured the lowest cytotoxicity and mechanical integrity of the restorative material. The composite that inhibited the formation of the *S. mutans* clusters on its surface exhibited less EPS and sparse bacterial aggregation. Zhang et al. [144] analyzed the antibacterial functions of dental adhesives containing dimethylaminododecyl methacrylate (DMADDM) on different bacteria. The result revealed that 5% DMADDM significantly decreased EPS levels in the biofilm matrix by reducing the content of the water-insoluble glucan in EPS.

4.2. CRISPR arrays

Due to its simplicity, high-efficiency, and specificity, CRISPR-associated enzyme (Cas) is a promising and competent genome editing tool that holds great promise for the treatment of incurable genetic disorders [145,146]. The CRISPR-Cas systems are primarily comprised of four components, including CRISPR regions, leader sequences, Cas proteins, and tracrRNA (for Cas9). CRISPR-Cas systems are primarily divided into two types based on the highly diverse *cas* genes and the mechanism of action in Cas proteins: (1) types I, III, and IV systems (multiplex Cas proteins); (2) types II, V, and VI systems (single Cas proteins) [147]. By cleaving DNA or selectively disrupting pathogenicity and virulence-associated genes, CRISPR-Cas may provide an excellent opportunity to combat microbial resistance (Fig. 6A) [148]. Zuberi et al. [149] introduced a unique concept known as “CRISPR interference (CRISPRi)”. In comparison with “knock-out” screens, CRISPRi inhibition provides varying levels of targeted knockdown, allowing for the investigation of behavioral changes in cells when the level of gene expression varies. To further inhibit biofilm formation, researchers used a CRISPR-derived CRISPR interference system to knock down the luxS gene, which plays an important role in the QS system in *E. coli*. According to the results of crystal violet staining and the 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) assay, the majority of cells were metabolically active, but biofilm formation was significantly decreased. One possible reason may be due to the reduction of EPS production, which disrupts the initial stage of biofilm formation. In another study, CRISPRi vectors (pPFL-dCas9) and deletion mutant strains were applied to *Pseudomonas fluorescens* (*P. fluorescens*) in order to investigate the role of genes involved in the GacA/S two-component system, enabling quantitative phenotyping analysis. After the silencing of the pleiotropic *gacS* (PFLU3777) gene with CRISPRi, confocal microscopy was used to image the established biofilm. The 3D reconstruction of biofilms revealed that *gacS* silencing contributed to a thinner and less biofilm compared to the control with irregular density and discontinuities. CRISPRi-mediated silencing is an efficient method to study gene-phenotype relationships in *P. fluorescens* [150].

Deletion of *gtfB* and *gtfC* is essential to achieve the most significant reduction in EPS and biofilm formation in *S. mutans*. Gong et al. [151] examined whether the assembly of the CRISPR Cas9 system from *S. mutans* UA159 was required for protection against exogenous DNA. It was observed that a suitable PAM site, tracrRNA, Cas9, and RNase III were essential components to display normal function in the *S. mutans* CRISPR Cas9 system. Researchers proposed the self-targeting CRISPR arrays (containing spacer sequences identifying with *gtfB*) and cloned them onto plasmids. Then the plasmids and editing templates were converted to UA159 (self-targeting) to obtain the required mutants. This technology can edit *gtfB* or *gtfB*/*gtfC* genes, resulting in a significant decrease in EPS synthesis and biofilm formation.

4.3. Enzymatic methods

Due to several limitations of antimicrobials, such as their low efficiency to combat mature biofilms, enzymatic methods can also be used as a supplement to chemical treatment protocols. Fig. 6B depicts how, during the biofilm dispersion stage, the bacteria in the biofilm must secrete extracellular enzymes to hydrolyze EPS components in order to degrade the structure of the biofilm and allow active diffusion of bacteria. Based on this principle, biofilm matrix-degrading enzymes can be used to design novel agents to enhance biofilm removal efficacy.

Based on their ability to conduct reactions with high environmental sensitivity and catalyze chemical reactions, enzymes have a promising therapeutic potential for treating infectious diseases [152]. For instance, the dissolution of the polysaccharides by enzymes could weaken the biofilm and render the sessile microbial population susceptible to antibiotics. This would ensure the elimination of pathogens from the infected areas [153].

4.3.1. Glycoside hydrolases

By degrading exopolysaccharides, glycoside hydrolases such as amylase, mannosidase, cellulase, and alginate lyase can disrupt the integrity of biofilm [154]. For example, α-amylase is used to modify manganese dioxide nanosheets (MNS) in order to enhance anti-biofilm efficacy of conventional sonodynamic therapy. After being incorporated into the biofilm, α-amylase is released locally, further degrading bacterial EPS. Due to the degradation of biofilm EPS, the modified therapeutic agent decorated with α-amylase presents a higher fluorescence intensity and depth, demonstrating the enhancement of sonosensitizer penetration and accumulation in the biofilm [155]. In addition, alginate lyase and ceftazidime loaded in mesoporous organosilica-coated Ag NPs (Ag@MON-AE) also provide a promising approach for combating biofilm-related infections. Alginate lyase can hydrolyze alginate, damage biofilm structure, and enhance the permeation of Ag@MON-AE into the biofilm [156].

4.3.2. Proteases and deoxyribonuclease

In addition to polysaccharides, proteins are the major components of EPS; therefore, proteases such as Spl protease and aureolysin may be capable of degrading EPS [157]. DNases and proteinases possess the ability to degrade EPS, thereby creating pathways for antimicrobials to penetrate the inner layers of the biofilm, where the most resistant microbial bacteria are colonized [158,159]. DNase II, DNase I, and TREX1 are the primary serum

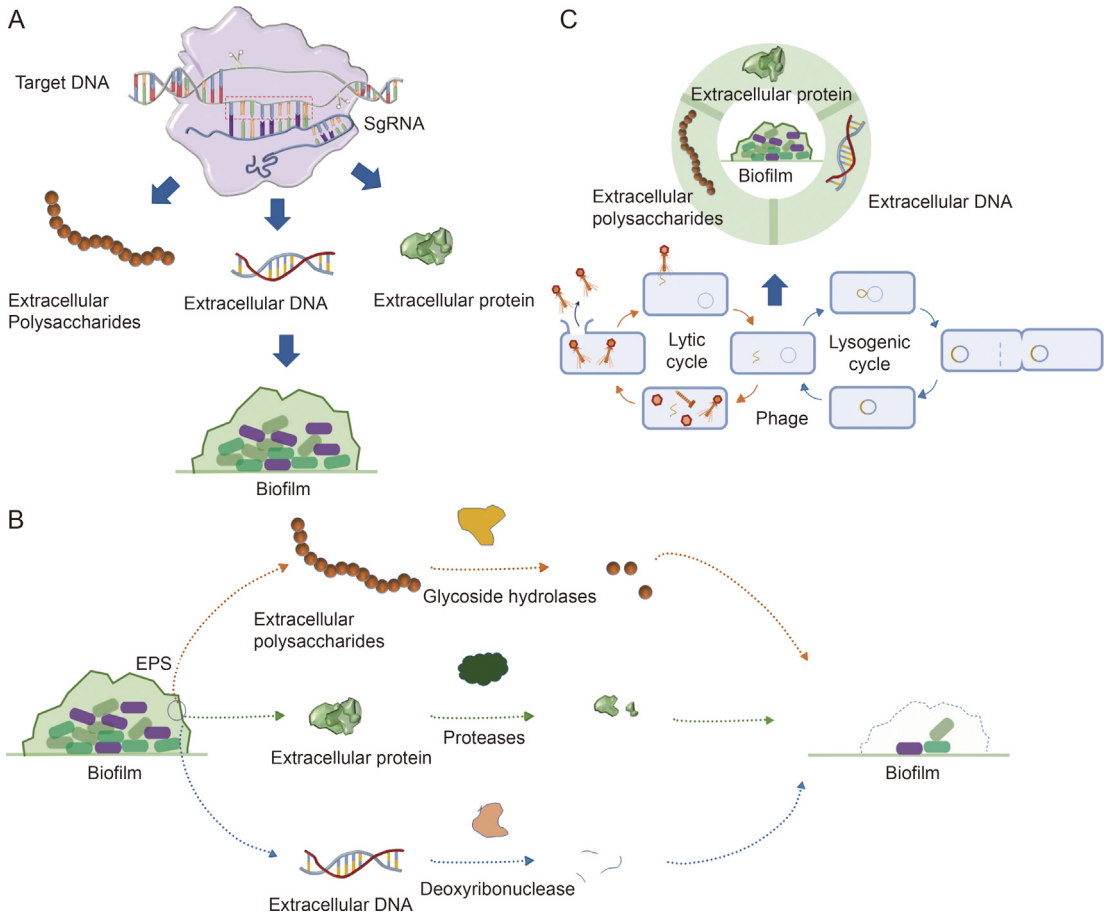

Fig. 6. Extracellular polymeric substances (EPS) targeting strategies using (A) clustered regularly interspaced short palindromic repeats (CRISPR) arrays, (B) enzymatic methods, and (C) phage therapy.

endonuclease-degrading eDNAs in the EPS [160]. Researchers investigated the effect of DNase I and proteinase K on the formation of a simulated supragingival biofilm [161]. Interestingly, there was no influence on the total bacterial quantities targeting the eDNA with DNase I in the multispecies biofilm matrix, while DNase I transformed the bacterial constituents and spatial pattern of the bacterial species in the biofilm. This phenomenon is likely attributed to the beneficial role of eDNA in facilitating the gene transfer processes [162]. It was observed that depredation of catalase (katA) could increase the level of ROS and increase susceptibility to peroxide [163–166]. The biofilm biomass decrement is due to the reduction in EPS production (including the decline of carbohydrate content) by the katA mutant. The deficiency in biofilm formation is pivotal, as it leads to a significant reduction in antibiotic resistance in vitro and a defect in colonization during experimental catheter-associated urinary tract infections (CAUTIs) [167].

Wen et al. [168] discovered that proteinase K, an extracellular matrix degradative agent, significantly eliminated the established biofilm of *Corynebacterium striatum* (*C. striatum*). In addition, proteinase K, in combination with lipase and cellulase, inhibit and eradicate *Vibrio parahaemolyticus* (*V. parahaemolyticus*) biofilm with remarkable efficacy. Combined enzymes reduce EPS formation, disrupt the EPS matrix network, and down-regulate the expression of the EPS-related gene *cpsA-J* [169].

Photodynamic therapy (PDAT) in combination with proteinase K was reported to have a significant antibiofilm effect. Zeolitic imidazolate framework-8 (ZIF-8) was used to encapsulate proteinase K and the photosensitizer Rose Bengal. Under an acidic environment, the proteinase K released by ZIF-8 caused the hydrolyzation of EPS and disruption of biofilm integrity, thereby promoting RB diffusion and accumulation in the biofilm. Under visible light activation, abundant ROS generated by RB were able to eradicate bacteria even inside the biofilm [170]. Based on a similar approach, Weldrick et al. [171] reported a protease-wrapped, ciprofloxacin-loaded nanogel. The alcalase 2.4 L FG protease can digest the EPS matrix and gain access to the EPS barrier. Therefore, a high dose of ciprofloxacin can be transmitted directly to the bacterial cell wall, thereby disrupting their defenses.

Inspired by the mechanism of EPS formation and dispersion, numerous studies have focused on EPS degradation by biological methods (e.g., the use of bioactive enzymes). However, the delivery of these enzymes must still overcome obstacles such as enzymes inactivation and inefficient delivery.

4.4. Phage therapy

Phages are one of the most ubiquitous organisms on earth, thriving in diverse habitats alongside specific host bacteria [172,173]. According to the life cycle of infection, phages can be divided into two types: lytic phages and lysogenic phages. After infecting the host bacteria, lytic phages (virulent phages) can rapidly replicate their genomes in a short time, leading to cell disruption and lysis [174]. Lysogenic phages (temperate phages), on the other hand, integrate their genome into the host genome as stable prophages without causing cell destruction. Interestingly, prophages can transfer into lytic states under environmental stimulation [175,176]. Phages are potential candidates for antibacterial therapy (Fig. 6C) due to their high specificity, affecting only target

microbes without causing non-toxicity to eukaryotic cells. Moreover, phage production is easy, rapid, and relatively inexpensive.

It has been reported that phages can utilize three unique mechanisms for the eradication of biofilms, including the production of EPS depolymerases, the lysis of bacteria forming biofilm (lysis from within), and "lysis from without", which does not require phage gene expression after absorption [177].

A few *in vitro* samples have demonstrated that phages can penetrate into the inner spaces of biofilms by degrading components of EPS, which results from the function of depolymerases induced by phages. An increasing number of publications have demonstrated that the application of phages has veterinary applications. However, phage therapy for human beings has been delayed due to a lack of consistent scientific evidence. Intriguingly, recent accumulated experimental evidence has aroused interest in phage therapy for humans, including the success of phage lysins against *streptococcal* and *pneumococal* infections [178] and a phage cocktail against purulent wound infections induced by five microbes [179].

Phages ISTD and NOVI were demonstrated to be potential candidates for combating *Acinetobacter baumannii* (*A. baumannii*) infections, as they can effectively reduce planktonic and biofilm-associated viable bacterial cell counts. The result of halo dynamics proved that phages can produce depolymerases to remove bacterial surface polysaccharides [180]. Similarly, phage DW-EC, which has high specificity for Enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), and Enterotoxigenic *E. coli* (ETEC), exhibits a remarkable antibiofilm effect. It produces lysine and putative T4-like lysozyme to disrupt and lyse cells, thereby preventing bacteria from attaining the required density to form EPS. In addition, mature biofilms are destroyed due to the degradation of bacterial polysaccharides caused by depolymerases [181].

In addition, it was discovered that Sb-1, a *S.aureus*-specific phage, degrades the methicillin-resistant *Staphylococcus aureus* (MRSA) polysaccharide matrix and eradicates biofilm in a dose-dependent manner. Notably, pretreatment with Sb-1 followed by the administration of rifampin/daptomycin at sub-MICs has a synergistic effect on eradicating MRSA biofilm [182].

4.5. Other approaches

Park et al. [183] investigated the effects of synthetic peptides, including PS1-2, PS1-5, and PS1-6, together with commonly used antibiotics, on biofilm formation and decrement in drug-resistant *P. aeruginosa* and *S.aureus*. The results revealed that all polypeptides inhibit planktonic bacteria growth and biofilm formation in a dose-dependent manner. The polypeptides reduced the predicted biofilm masses by decreasing the presence of carbohydrates, eDNA, and lipids within the EPS. Interestingly, the content of proteins remained unaffected in the presence of polypeptides.

The extracytoplasmic function (ECF) sigma factor AlgU has been observed to play a significant role in EPS secretion and biofilm formation in *P. stutzeri* A1501 [184]. The transcriptional level of *pslA*, a critical Psl-like (a primary EPS in A1501) gene interrelated to synthesis, is completely suppressed in the *algU* mutant and is upregulated by 2.8-fold in the *algU*-overexpressing strain. The DNase I footprinting experiments revealed that AlgU could successfully combine with the *pslA* promoter, and β-galactosidase activation analysis also revealed mutations of the AlgU-binding boxes decreased the transcriptional activity of the *pslA* promoter. By directly activating the transcription of *pslA*, AlgU stimulates EPS secretion and biofilm formation. These results indicate that the deletion of AlgU provides a promising strategy to suppress EPS production in *P. stutzeri*. In a similar manner, a small diffusible molecule known as DSF activates the expression of the *manA* gene, responsible for encoding endo-β-1, 4-mannanase. This activation leads to a reduction in both EPS and biofilm formation [185].

Rhamnolipids, microbial-synthesized biosurfactants, were first reported to be connected with *P. aeruginosa* biofilms [186]. Interestingly, adequate concentrations (<0.4 g/L) of rhamnolipid are vital for the maintenance of mature biofilms; however, for a variety of bacterial species, excessive rhamnolipid concentrations can result in biofilm spread [186–188].

5. Conclusions and outlook

Biofilms that result in persistent bacterial infections pose serious threats to human health as the EPS matrix of the biofilm prevents the diffusion of integral antimicrobials. The limited ability of antimicrobials to infiltrate biofilms significantly diminishes their effectiveness in eradicating bacteria [142]. The key to eliminating bacterial biofilm is to overcome the impediment of EPS produced by the bacteria and facilitate the permeation of antimicrobials into the biofilm. Consequently, overcoming the hindrance of EPS is a critical point in designing and developing antibiofilm therapeutic agents.

At present, the characterization of EPS constituents is regarded as an important aspect of numerous studies concerning antibiofilm agents; however, the majority of these studies focus solely on detecting the levels of EPS components without elucidating the signaling pathways involved in the synthesis and transformation of polysaccharides and other components. Furthermore, these studies largely relied on colorimetric approaches to quantify EPS constituents, ignoring the inaccuracy caused by the deviation of the method and thereby increasing the uncertainty of the anti-biofilm effect. Especially when treating the biofilm with plant-derived agents, the interference of exogenous plant components became more significant. Current analysis of EPS composition largely depends on colorimetric approaches with a significant bias, which is likely due to the selection of a standard compound and the cross-interference of various EPS compounds within each assay. In view of the importance of EPS to the functioning of biofilm, EPS research is expected to delve deeper into the analysis of complex compositions beyond relying extensively on colorimetric analytical methods.

The molecular weight, structure, surface properties, morphology, and other physical and chemical properties of antibiofilm therapeutic agents should be considered when designing and developing antibiofilm therapeutic agents in an effort to enhance drug penetration of these agents into biofilms. Furthermore, targeted delivery by electrostatic interactions or functional molecular modifications can partially surmount biofilm barriers and increase the penetrability of antibiofilm agents. In addition to these targeting strategies, nano/micromotors, magnetic drives, and other strategies that use an external force to propel antibacterial or antibiofilm agents into the inner biofilm are also emerging. For instance, micro/nanorobots that are powered by versatile sources such as light, magnetic fields, and thermal energy, work like motors to promote rapid drug delivery by performing specific mechanical actions.

Finally, although the future use of EPS inhibitors for the treatment of medical biofilms is extremely promising, much of the current evaluation of enhanced biofilm penetrability was conducted in vitro. Few clinical experiments, or even in vivo research, particularly in large mammals, have been conducted. The translation of the numerous early experimental achievements into clinical applications must also progress. Furthermore, it is imperative to explore more realistic biofilm models that closely approximate the properties of biofilm in clinical infections. In conclusion, the pursuit of EPS inhibitors and the

investigation of their inhibitory mechanisms require unwavering perseverance.

CRediT author statement

Lan Lu: Conceptualization, Writing - Original draft preparation, Project administration; **Yuting Zhao:** Writing - Original draft preparation, Resources, Investigation; **Mingxing Li:** Investigation, Resources; **Xiaobo Wang:** Investigation, Resources; **Jie Zhu:** Investigation, Conceptualization, Project administration; **Li Liao:** Data curation, Resources; **Jingya Wang:** Conceptualization, Writing - Original draft preparation.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was funded by the National Natural Science Foundation of China (Grant Nos.: 81803812 and 81803237).

References

[1] J. Hurlow, K. Couch, K. Laforet, et al., Clinical biofilms: A challenging frontier in wound care, Adv. Wound Care 4 (2015) 295–301.
[2] L. Lu, Y. Zhao, G. Yi, et al., Quinic acid: A potential antibiofilm agent against clinical resistant *Pseudomonas aeruginosa*, Chin. Med. 16 (2021), 72.
[3] W. Zhang, J. Zhang, K. Zhao, Application of bacterial tracking techniques in biofilms, Sheng Wu Gong Cheng, Xue Bao 33 (2017) 1411–1432.
[4] H.C. Flemming, J. Wingender, The biofilm matrix, Nat. Rev. Microbiol. 8 (2010) 623–633.
[5] H. van Acker, P. van Dijck, T. Coenye, Molecular mechanisms of antimicrobial tolerance and resistance in bacterial and fungal biofilms, Trends Microbiol. 22 (2014) 326–333.
[6] S.S. Branda, S. Vik, L. Friedman, et al., Biofilms: The matrix revisited, Trends Microbiol 13 (2005) 20–26.
[7] M. Ganesan, E.J. Stewart, J. Szafranski, et al., Molar mass, entanglement, and associations of the biofilm polysaccharide of *Staphylococcus epidermidis*, Biomacromolecules 14 (2013) 1474–1481.
[8] A. Dragoš, Á.T. Kovács, The peculiar functions of the bacterial extracellular matrix, Trends Microbiol 25 (2017) 257–266.
[9] H. Koo, R.N. Allan, R.P. Howlin, et al., Targeting microbial biofilms: Current and prospective therapeutic strategies, Nat. Rev. Microbiol. 15 (2017) 740–755.
[10] L. Karygianni, Z. Ren, H. Koo, et al., Biofilm matrixome: Extracellular components in structured microbial communities, Trends Microbiol 28 (2020) 668–681.
[11] H.C. Flemming, J. Wingender, U. Szewzyk, et al., Biofilms: An emergent form of bacterial life, Nat. Rev. Microbiol. 14 (2016) 563–575.
[12] J.A. Bartell, L.M. Sommer, J.A.J. Haagensen, et al., Evolutionary highways to persistent bacterial infection, Nat. Commun. 10 (2019), 629.
[13] B.H.A. Rehm, Bacterial polymers: Biosynthesis, modifications and applications, Nat. Rev. Microbiol. 8 (2010) 578–592.
[14] J. Schmid, V. Sieber, B. Rehm, Bacterial exopolysaccharides: Biosynthesis pathways and engineering strategies, Front. Microbiol. 6 (2015), 496.
[15] H. Shen, D.P. Durkin, A. Aiello, et al., Photocatalytic graphitic carbon nitride-chitosan composites for pathogenic biofilm control under visible light irradiation, J. Hazard. Mater. 408 (2021), 124890.
[16] J. Wingender, M. Strathmann, A. Rode, et al., Isolation and biochemical characterization of extracellular polymeric substances from *Pseudomonas aeruginosa*, Meth. Enzymol. 336 (2001) 302–314.
[17] P.M. Bales, E.M. Renke, S.L. May, et al., Purification and characterization of biofilm-associated EPS exopolysaccharides from ESKAPE organisms and other pathogens, PLoS One 8 (2013), e67950.
[18] D.H. Limoli, C.J. Jones, D.J. Wozniak, Bacterial extracellular polysaccharides in biofilm formation and function, Microbiol. Spectr. 3 (2015), https://doi.org/10.1128/microbiolspec.MB-0011-2014.
[19] C. Watters, D. Fleming, D. Bishop, et al., Host responses to biofilm, Prog. Mol. Biol. Transl. Sci. 142 (2016) 193–239.
[20] Q. Wang, F. Kang, Y. Gao, et al., Sequestration of nanoparticles by an EPS matrix reduces the particle-specific bactericidal activity, Sci. Rep. 6 (2016), 21379.
[21] J.W. Lamppa, K.E. Griswold, Alginate lyase exhibits catalysis-independent biofilm dispersion and antibiotic synergy, Antimicrob. Agents Chemother. 57 (2013) 137–145.
[22] D. Fleming, L. Chahin, K. Rumbaugh, Glycoside hydrolases degrade polymicrobial bacterial biofilms in wounds, Antimicrob. Agents Chemother. 61 (2017), e01998–e01916.
[23] P. Baker, P.J. Hill, B.D. Snarr, et al., Exopolysaccharide biosynthetic glycoside hydrolases can be utilized to disrupt and prevent *Pseudomonas aeruginosa* biofilms, Sci Adv 2 (2016), e1501632.
[24] E.A. Izano, M.A. Amarante, W.B. Kher, et al., Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms, Appl. Environ. Microbiol. 74 (2008) 470–476.
[25] I. Līduma, T. Tračevska, U. Bērs, et al., Phenotypic and genetic analysis of biofilm formation by *Staphylococcus epidermidis*, Med. Kaunas Lith. 48 (2012) 305–309.
[26] K.M. Conlon, H. Humphreys, J.P. O'Gara, icaR encodes a transcriptional repressor involved in environmental regulation of Ica operon expression and biofilm formation in *Staphylococcus epidermidis*, J. Bacteriol. 184 (2002) 4400–4408.
[27] Y. Mu, H. Zeng, W. Chen, Quercetin inhibits biofilm formation by decreasing the production of EPS and altering the composition of EPS in *Staphylococcus epidermidis*, Front. Microbiol. 12 (2021), 631058.
[28] M.I. Klein, G. Hwang, P.H.S. Santos, et al., *Streptococcus mutans*-derived extracellular matrix in cariogenic oral biofilms, Front. Cell. Infect. Microbiol. 5 (2015), 10.
[29] W.H. Bowen, H. Koo, Biology of *Streptococcus mutans*-derived glucosyltransferases: Role in extracellular matrix formation of cariogenic biofilms, Caries Res. 45 (2011) 69–86.
[30] L. Guo, J.S. McLean, R. Lux, et al., The well-coordinated linkage between acidogenicity and aciduricity via insoluble glucans on the surface of *Streptococcus mutans*, Sci. Rep. 5 (2015) 18015.
[31] W.L. Kuhnert, G. Zheng, R.C. Faustoferri, et al., The F-ATPase operon promoter of Streptococcus *mutans* is transcriptionally regulated in response to external pH, J. Bacteriol. 186 (2004) 8524–8528.
[32] Z. Ren, L. Chen, J. Li, et al., Inhibition of *Streptococcus mutans* polysaccharide synthesis by molecules targeting glycosyltransferase activity, J. Oral Microbiol. 8 (2016), 31095.
[33] G.R. Rocha, E.J. Florez Salamanca, A.L. de Barros, et al., Effect of tt-farnesol and myricetin on *in vitro* biofilm formed by *Streptococcus mutans* and *Candida albicans*, BMC Complement. Altern. Med. 18 (2018), 61.
[34] R.K. Upreti, M. Kumar, V. Shankar, Bacterial glycoproteins: Functions, biosynthesis and applications, Proteomics 3 (2003) 363–379.
[35] M.F. Moradali, B.H.A. Rehm, Bacterial biopolymers: From pathogenesis to advanced materials, Nat. Rev. Microbiol. 18 (2020) 195–210.
[36] U.T. Nguyen, L.L. Burrows, DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms, Int. J. Food Microbiol. 187 (2014) 26–32.
[37] S. Kumar Shukla, T.S. Rao, Dispersal of bap-mediated *Staphylococcus aureus* biofilm by proteinase K, J. Antibiot. 66 (2013) 55–60.
[38] J.B. Kaplan, Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses, J. Dent. Res. 89 (2010) 205–218.
[39] X. Zhang, P.L. Bishop, Biodegradability of biofilm extracellular polymeric substances, Chemosphere 50 (2003) 63–69.
[40] L. Hobley, C. Harkins, C.E. MacPhee, et al., Giving structure to the biofilm matrix: An overview of individual strategies and emerging common themes, FEMS Microbiol. Rev. 39 (2015) 649–669.
[41] I. Lasa, J.R. Penadés, Bap: A family of surface proteins involved in biofilm formation, Res. Microbiol. 157 (2006) 99–107.
[42] L.P. Blanco, M.L. Evans, D.R. Smith, et al., Diversity, biogenesis and function of microbial amyloids, Trends Microbiol 20 (2012) 66–73.
[43] D.M. Fowler, A.V. Koulov, W.E. Balch, et al., Functional amyloid: From bacteria to humans, Trends Biochem. Sci. 32 (2007) 217–224.
[44] D. Romero, E. Sanabria-Valentín, H. Vlamakis, et al., Biofilm inhibitors that target amyloid proteins, Chem. Biol. 20 (2013) 102–110.
[45] L. Shaw, E. Golonka, J. Potempa, et al., The role and regulation of the extracellular proteases of *Staphylococcus aureus*, Microbiol. Read. Engl. 150 (2004) 217–228.
[46] A.J. Loughran, D.N. Atwood, A.C. Anthony, et al., Impact of individual extracellular proteases on *Staphylococcus aureus* biofilm formation in diverse clinical isolates and their isogenic sarA mutants, MicrobiologyOpen 3 (2014) 897–909.
[47] M. Martí, M.P. Trotonda, M.A. Tormo-Más, et al., Extracellular proteases inhibit protein-dependent biofilm formation in *Staphylococcus aureus*, Microbes Infect 12 (2010) 55–64.
[48] M. Gjermansen, M. Nilsson, L. Yang, et al., Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms: Genetic elements and molecular mechanisms, Mol. Microbiol. 75 (2010) 815–826.
[49] B.R. Boles, A.R. Horswill, Agr-mediated dispersal of *Staphylococcus aureus* biofilms, PLoS Pathog 4 (2008), e1000052.
[50] K.J. Lauderdale, B.R. Boles, A.L. Cheung, et al., Interconnections between *Sigma* B, agr, and proteolytic activity in *Staphylococcus aureus* biofilm maturation, Infect. Immun. 77 (2009) 1623–1635.
[51] D.C. Nelson, J. Garbe, M. Collin, Cysteine proteinase SpeB from *Streptococcus pyogenes*—a potent modifier of immunologically important host and bacterial proteins, Biol. Chem. 392 (2011) 1077–1088.

[52] K.L. Connolly, A.L. Roberts, R.C. Holder, et al., Dispersal of Group A streptococcal biofilms by the cysteine protease SpeB leads to increased disease severity in a murine model, PLoS One 6 (2011), e18984.
[53] S.F. Lee, Y.H. Li, G.H. Bowden, Detachment of *Streptococcus mutans* biofilm cells by an endogenous enzymatic activity, Infect. Immun. 64 (1996) 1035–1038.
[54] M. Banar, M. Emaneini, M. Satarzadeh, et al., Evaluation of mannosidase and trypsin enzymes effects on biofilm production of *Pseudomonas aeruginosa* isolated from burn wound infections, PLoS One 11 (2016), e0164622.
[55] S.A. Niazi, D. Clark, et al., The effectiveness of enzymic irrigation in removing a nutrient-stressed endodontic multispecies biofilm, Int. Endod. J. 47 (2014) 756–768.
[56] M. Okshevsky, R.L. Meyer, The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms, Crit. Rev. Microbiol. 41 (2015) 341–352.
[57] M. Alhede, T. Bjarnsholt, M. Givskov, et al., *Pseudomonas aeruginosa* biofilms: Mechanisms of immune evasion, Adv. Appl. Microbiol. 86 (2014) 1–40.
[58] N.S. Jakubovics, R.C. Shields, N. Rajarajan, et al., Life after death: The critical role of extracellular DNA in microbial biofilms, Lett. Appl. Microbiol. 57 (2013) 467–475.
[59] M. Allesen-Holm, K.B. Barken, L. Yang, et al., A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms, Mol. Microbiol. 59 (2006) 1114–1128.
[60] L. Lu, M. Li, G. Yi, et al., Screening strategies for quorum sensing inhibitors in combating bacterial infections, J. Pharm. Anal. 12 (2022) 1–14.
[61] J.S. Webb, L.S. Thompson, S. James, et al., Cell death in *Pseudomonas aeruginosa* biofilm development, J. Bacteriol. 185 (2003) 4585–4592.
[62] L.K. Jennings, K.M. Storek, H.E. Ledvina, et al., Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 11353–11358.
[63] M.F. Moradali, S. Ghods, B.H. Rehm, *Pseudomonas aeruginosa* lifestyle: A paradigm for adaptation, survival, and persistence, Front. Cell. Infect. Microbiol. 7 (2017), 39.
[64] J. Fang, Y. Lin, P. Wang, et al., The droplet-size effect of squalene@cetylpyridinium chloride nanoemulsions on antimicrobial potency against planktonic and biofilm MRSA, Int. J. Nanomed. 14 (2019) 8133–8147.
[65] M. Dubois, K. Gilles, J.K. Hamilton, et al., A colorimetric method for the determination of sugars, Nature 168 (1951) 167.
[66] O.H. Lowry, N.J. Rosebrough, A.L. Farr, et al., Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[67] Z. Dische, A new specific color reaction of hexuronic acids, J. Biol. Chem. 167 (1947) 189–198.
[68] J. Kang, L. Liu, Y. Liu, et al., Ferulic acid inactivates *Shigella flexneri* through cell membrane destruction, biofilm retardation, and altered gene expression, J. Agric. Food Chem. 68 (2020) 7121–7131.
[69] A.T. Bernal-Mercado, M.M. Gutierrez-Pacheco, D. Encinas-Basurto, et al., Synergistic mode of action of catechin, vanillic and protocatechuic acids to inhibit the adhesion of uropathogenic *Escherichia coli* on silicone surfaces, J. Appl. Microbiol. 128 (2020) 387–400.
[70] A. Minich, Z. Levarski, M. Mikulášová, et al., Complex analysis of vanillin and syringic acid as natural antimicrobial agents against *Staphylococcus epidermidis* biofilms, Int. J. Mol. Sci. 23 (2022), 1816.
[71] F. Liu, P. Jin, H. Gong, et al., Antibacterial and antibiofilm activities of thyme oil against foodborne multiple antibiotics-resistant *Enterococcus faecalis*, Poult. Sci. 99 (2020) 5127–5136.
[72] D. Rubini, S.F. Banu, P. Nisha, et al., Essential oils from unexplored aromatic plants quench biofilm formation and virulence of Methicillin resistant *Staphylococcus aureus*, Microb. Pathog. 122 (2018) 162–173.
[73] Y. Liu, Y. Xu, Q. Song, et al., Anti-biofilm activities from *Bergenia crassifolia* leaves against *Streptococcus mutans*, Front. Microbiol. 8 (2017) 1738.
[74] J. Li, Q. Fan, M. Jin, et al., Paeoniflorin reduce *luxS*/AI-2 system-controlled biofilm formation and virulence in *Streptococcus suis*, Virulence 12 (2021) 3062–3073.
[75] H.S. Kim, H.D. Park, Ginger extract inhibits biofilm formation by *Pseudomonas aeruginosa* PA14, PLoS One 8 (2013), e76106.
[76] V. Tiwari, D. Tiwari, V. Patel, et al., Effect of secondary metabolite of *Actinidia deliciosa* on the biofilm and extra-cellular matrix components of *Acinetobacter baumannii*, Microb. Pathog. 110 (2017) 345–351.
[77] R. Srinivasan, S. Santhakumari, A.V. Ravi, *In vitro* antibiofilm efficacy of *Piper betle* against quorum sensing mediated biofilm formation of luminescent *Vibrio harveyi*, Microb. Pathog. 110 (2017) 232–239.
[78] M. Majumdar, A. Dubey, R. Goswami, et al., *In vitro* and in silico studies on the structural and biochemical insight of anti-biofilm activity of andrographanin from *Andrographis paniculata* against *Pseudomonas aeruginosa*, World J. Microbiol. Biotechnol. 36 (2020), 143.
[79] S. Xing, X. Sun, A.A. Taylor, et al., D-amino acids inhibit initial bacterial adhesion: Thermodynamic evidence, Biotechnol. Bioeng. 112 (2015) 696–704.
[80] H. Xu, Y. Liu, Reduced microbial. attachment by D-amino acid-inhibited AI-2 and EPS production, Water Res 45 (2011) 5796–5804.
[81] Y. Liu, Y. Jiang, J. Zhu, et al., Inhibition of bacterial adhesion and biofilm formation of sulfonated chitosan against *Pseudomonas aeruginosa*, Carbohydr. Polym. 206 (2019) 412–419.
[82] R.A. Akeel, H.F. Hetta, S.N. Muslim, et al., Broad-spectrum bioactivity of chitosan N-acetylglucosaminohydrolase (chitosan NAGH) extracted from *Bacillus ligniniphilus*, J. AOAC Int. 102 (2019) 1221–1227.
[83] M. Kalia, V.K. Yadav, P.K. Singh, et al., Effect of cinnamon oil on quorum sensing-controlled virulence factors and biofilm formation in *Pseudomonas aeruginosa*, PLoS One 10 (2015), e0135495.
[84] L. Lin, W. Jianhuit, Y. Jialini, et al., Effects of allicin on the formation of *Pseudomonas aeruginosa* biofinm and the production of quorum-sensing controlled virulence factors, Pol. J. Microbiol. 62 (2013) 243–251.
[85] J. Tao, S. Yan, C. Zhou, et al., Total flavonoids from *Potentilla kleiniana* Wight et Arn inhibits biofilm formation and virulence factors production in methicillin-resistant *Staphylococcus aureus* (MRSA), J. Ethnopharmacol. 279 (2021) 114383.
[86] C. Yu, X. Li, N. Zhang, et al., Inhibition of biofilm formation by D-tyrosine: Effect of bacterial type and D-tyrosine concentration, Water Res. 92 (2016) 173–179.
[87] T.R. Neu, J.R. Lawrence, Lectin-binding analysis in biofilm systems, Methods Enzymol 310 (1999) 145–152.
[88] M. Strathmann, J. Wingender, H.C. Flemming, Application of fluorescently labelled lectins for the visualization and biochemical characterization of polysaccharides in biofilms of *Pseudomonas aeruginosa*, J. Microbiol. Methods 50 (2002) 237–248.
[89] J.R. Lawrence, G.D.W. Swerhone, U. Kuhlicke, et al., *In situ* evidence for microdomains in the polymer matrix of bacterial microcolonies, Can. J. Microbiol. 53 (2007) 450–458.
[90] R.Y. Zhang, T.R. Neu, S. Bellenberg, et al., Use of lectins to *in situ* visualize glycoconjugates of extracellular polymeric substances in acidophilic archaeal biofilms, Microb. Biotechnol. 8 (2015) 448–461.
[91] R. Zhang, T.R. Neu, Y. Zhang, et al., Visualization and analysis of EPS glycoconjugates of the thermoacidophilic archaeon *Sulfolobus metallicus*, Appl. Microbiol. Biotechnol. 99 (2015) 7343–7356.
[92] K. Rebrosova, O. Samek, M. Kizovsky, et al., Raman spectroscopy-a novel method for identification and characterization of microbes on a single-cell level in clinical settings, Front. Cell. Infect. Microbiol. 12 (2022), 866463.
[93] Y. Shang, M. Sun, C. Wang, et al., Research progress in distributed acoustic sensing techniques, Sensors (Basel) 22 (2022), 6060.
[94] K.V. Serebrennikova, A.N. Berlina, D.V. Sotnikov, et al., Raman scattering-based biosensing: New prospects and opportunities, Biosensors 11 (2021), 512.
[95] A. Saletnik, B. Saletnik, C. Puchalski, Overview of popular techniques of Raman spectroscopy and their potential in the study of plant tissues, Molecules 26 (2021), 1537.
[96] B. Gieroba, M. Krysa, K. Wojtowicz, et al., The FT-IR and Raman spectroscopies as tools for biofilm characterization created by cariogenic streptococci, Int. J. Mol. Sci. 21 (2020), 3811.
[97] L. Tan, F. Zhao, Q. Han, et al., High correlation between structure development and chemical variation during biofilm formation by *Vibrio parahaemolyticus*, Front. Microbiol. 9 (2018), 1881.
[98] L.M. Malard, L. Lafeta, R.S. Cunha, et al., Studying 2D materials with advanced Raman spectroscopy: CARS, SRS and TERS, Phys. Chem. Chem. Phys. 23 (2021) 23428–23444.
[99] Y. Qiu, C. Kuang, X. Liu, et al., Single-molecule surface-enhanced Raman spectroscopy, Sensors (Basel) 22 (2022), 4889.
[100] H.X. Wang, Y.W. Zhao, Z. Li, et al., Development and application of aptamer-based surface-enhanced Raman spectroscopy sensors in quantitative analysis and biotherapy, Sensors (Basel) 19 (2019), 3806.
[101] N.P. Ivleva, M. Wagner, H. Horn, et al., Raman microscopy and surface-enhanced Raman scattering (SERS) for *in situ* analysis of biofilms, J. Biophotonics 3 (2010) 548–556.
[102] S.K. Pirutin, S. Jia, A.I. Yusipovich, et al., Vibrational spectroscopy as a tool for bioanalytical and biomonitoring studies, Int. J. Mol. Sci. 24 (2023), 6947.
[103] S. Yang, Q. Zhang, H. Yang, et al., Progress in infrared spectroscopy as an efficient tool for predicting protein secondary structure, Int. J. Biol. Macromol. 206 (2022) 175–187.
[104] F. Zhang, Q. Huang, Characterization of Z-DNA by infrared spectroscopy, Methods Mol. Biol. 2651 (2023) 53–58.
[105] K. Kaczmarek, A. Leniart, B. Lapinska, et al., Selected spectroscopic techniques for surface analysis of dental materials: A narrative review, Materials 14 (2021), 2624.
[106] Y. Alqaheem, A.A. Alomair, Microscopy and spectroscopy techniques for characterization of polymeric membranes, Membranes 10 (2020), 33.
[107] F.S. Ghoreishi, R. Roghanian, G. Emtiazi, Inhibition of quorum sensing-controlled virulence factors with natural substances and novel protease, obtained from *Halobacillus karajensis*, Microb. Pathog. 149 (2020), 104555.
[108] S. Felz, P. Vermeulen, M.C.M. van Loosdrecht, et al., Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS), Water Res. 157 (2019) 201–208.
[109] M.S. Byrd, I. Sadovskaya, E. Vinogradov, et al., Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production, Mol. Microbiol. 73 (2009) 622–638.

[110] Y. Cui, Y. Li, S. Jiang, et al., Isolation, purification, and structural characterization of polysaccharides from Atractylodis Macrocephalae Rhizoma and their immunostimulatory activity in $RAW_{264.7}$ cells, Int. J. Biol. Macromol. 163 (2020) 270–278.
[111] X. Gao, J. Qi, C.T. Ho, et al., Structural characterization and immunomodulatory activity of a water-soluble polysaccharide from *Ganoderma leucocontextum* fruiting bodies, Carbohydr. Polym. 249 (2020), 116874.
[112] L. Shi, Bioactivities, isolation and purification methods of polysaccharides from natural products: A review, Int. J. Biol. Macromol. 92 (2016) 37–48.
[113] V.P. Chakka, T. Zhou, Carboxymethylation of polysaccharides: Synthesis and bioactivities, Int. J. Biol. Macromol. 165 (2020) 2425–2431.
[114] M. Inagaki, R. Iwakuma, S. Kawakami, et al., Detecting and differentiating monosaccharide enantiomers by ^{1}H NMR spectroscopy, J. Nat. Prod. 84 (2021) 1863–1869.
[115] G. Nagy, N.L.B. Pohl, Monosaccharide identification as a first step toward *de novo* carbohydrate sequencing: Mass spectrometry strategy for the identification and differentiation of diastereomeric and enantiomeric pentose isomers, Anal. Chem. 87 (2015) 4566–4571.
[116] S. Zhou, G. Huang, G. Chen, Extraction, structural analysis, derivatization and antioxidant activity of polysaccharide from Chinese yam, Food Chem 361 (2021), 130089.
[117] F. An, G. Ren, J. Wu, et al., Extraction, purification, structural characterization, and antioxidant activity of a novel polysaccharide from *Lonicera japonica* Thunb, Front. Nutr. 9 (2022), 1035760.
[118] T. Mori, T. Kumano, H. He, et al., C-Glycoside metabolism in the gut and in nature: Identification, characterization, structural analyses and distribution of C-C bond-cleaving enzymes, Nat. Commun. 12 (2021), 6294.
[119] G. Liu, J. Ye, W. Li, et al., Extraction, structural characterization, and immunobiological activity of ABP Ia polysaccharide from *Agaricus bisporus*, Int. J. Biol. Macromol. 162 (2020) 975–984.
[120] B.A. Wallace, The role of circular dichroism spectroscopy in the era of integrative structural biology, Curr. Opin. Struct. Biol. 58 (2019) 191–196.
[121] L. Lu, W. Hu, Z. Tian, et al., Developing natural products as potential antibiofilm agents, Chin. Med. 14 (2019), 11.
[122] S.A. Lakshmi, J.P. Bhaskar, V. Krishnan, et al., Inhibition of biofilm and biofilm-associated virulence factor production in methicillin-resistant *Staphylococcus aureus* by docosanol, J. Biotechnol. 317 (2020) 59–69.
[123] H.M. Tran, H. Tran, M.A. Booth, et al., Nanomaterials for treating bacterial biofilms on implantable medical devices, Nanomaterials 10 (2020), 2253.
[124] W. Xiu, S. Gan, Q. Wen, et al., Biofilm microenvironment-responsive nanotheranostics for dual-mode imaging and hypoxia-relief-enhanced photodynamic therapy of bacterial infections, Research Wash. D C 2020 (2020), 9426453.
[125] J.A. Kloepfer, R.E. Mielke, J.L. Nadeau, Uptake of CdSe and CdSe/ZnS quantum dots into bacteria via purine-dependent mechanisms, Appl. Environ. Microbiol. 71 (2005) 2548–2557.
[126] J. Cao, Y. Zhao, Y. Liu, et al., Phosphorylcholine-based polymer encapsulated chitosan nanoparticles enhance the penetration of antimicrobials in a staphylococcal biofilm, ACS Macro Lett. 8 (2019) 651–657.
[127] M. Altaf, S. Manoharadas, M.T. Zeyad, Green synthesis of cerium oxide nanoparticles using *Acorus calamus* extract and their antibiofilm activity against bacterial pathogens, Microsc. Res. Tech. 84 (2021) 1638–1648.
[128] K. Ali, B. Ahmed, M.S. Khan, et al., Differential surface contact killing of pristine and low EPS *Pseudomonas aeruginosa* with *Aloe* vera capped hematite (α-Fe_2O_3) nanoparticles, J. Photochem. Photobiol. B 188 (2018) 146–158.
[129] P.C. Naha, Y. Liu, G. Hwang, et al., Dextran-coated iron oxide nanoparticles as biomimetic catalysts for localized and pH-activated biofilm disruption, ACS Nano 13 (2019) 4960–4971.
[130] S. Priyadarsini, S. Mukherjee, M. Mishra, Nanoparticles used in dentistry: A review, J. Oral Biol. Craniofac. Res. 8 (2018) 58–67.
[131] M.A. Dos Santos Ramos, P.B. Da Silva, L. Spósito, et al., Nanotechnology-based drug delivery systems for control of microbial biofilms: A review, Int. J. Nanomedicine 13 (2018) 1179–1213.
[132] J. Rajkumari, S. Busi, A.C. Vasu, et al., Facile green synthesis of baicalein fabricated gold nanoparticles and their antibiofilm activity against *Pseudomonas aeruginosa* PAO1, Microb. Pathog. 107 (2017) 261–269.
[133] B. Gayani, A. Dilhari, G.K. Wijesinghe, et al., Effect of natural curcuminoids-intercalated layered double hydroxide nanohybrid against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*: Abactericidal, antibiofilm, and mechanistic study, MicrobiologyOpen 8 (2019), e00723.
[134] J. Cai, H. Huang, W. Song, et al., Preparation and evaluation of lipid polymer nanoparticles for eradicating H. pylori biofilm and impairing antibacterial resistance *in vitro*, Int. J. Pharm. 495 (2015) 728–737.
[135] A. Tucak, M. Sirbubalo, L. Hindija, et al., Microneedles: Characteristics, materials, production methods and commercial development, Micromachines 11 (2020), 961.
[136] N.N. Al-Rawi, M. Rawas-Qalaji, Dissolving microneedles with antibacterial functionalities: A systematic review of laboratory studies, Eur. J. Pharm. Sci. 174 (2022), 106202.
[137] H. Shen, E.A. López-Guerra, R. Zhu, et al., Visible-light-responsive photocatalyst of graphitic carbon nitride for pathogenic biofilm control, ACS Appl. Mater. Interfaces 11 (2019) 373–384.
[138] S. Tian, L. Su, Y. Liu, et al., Self-targeting, zwitterionic micellar dispersants enhance antibiotic killing of infectious biofilms – an intravital imaging study in mice, Sci. Adv. 6 (2020), eabb1112.
[139] Y. Shi, J. Zhao, H. Li, et al., A drug-free, hair follicle cycling regulatable, separable, antibacterial microneedle patch for hair regeneration therapy, Adv. Healthc. Mater. 11 (2022), e2200908.
[140] K.N. Mangang, P. Thakran, J. Halder, et al., PVP-microneedle array for drug delivery: Mechanical insight, biodegradation, and recent advances, J. Biomater. Sci. Polym. Ed. 34 (2023) 986–1017.
[141] J. Fang, W. Chou, C.F. Lin, et al., Facile biofilm penetration of cationic liposomes loaded with DNase I/proteinase K to eradicate *Cutibacterium acnes* for treating cutaneous and catheter infections, Int. J. Nanomed. 16 (2021) 8121–8138.
[142] L. Xu, C.A. Siedlecki, *Staphylococcus epidermidis* adhesion on hydrophobic and hydrophilic textured biomaterial surfaces, Biomed. Mater. Bristol Engl. 9 (2014), 035003.
[143] G. Hwang, B. Koltisko, X. Jin, et al., Nonleachable imidazolium-incorporated composite for disruption of bacterial clustering, exopolysaccharide-matrix assembly, and enhanced biofilm removal, ACS Appl. Mater. Interfaces 9 (2017) 38270–38280.
[144] K. Zhang, S. Wang, X. Zhou, et al., Effect of antibacterial dental adhesive on multispecies biofilms formation, J. Dent. Res. 94 (2015) 622–629.
[145] B. Zhang, CRISPR/Cas gene therapy, J. Cell. Physiol. 236 (2021) 2459–2481.
[146] G. Sharma, A.R. Sharma, M. Bhattacharya, et al., CRISPR-Cas9: A preclinical and clinical perspective for the treatment of human diseases, Mol. Ther. 29 (2021) 571–586.
[147] G. Liu, Q. Lin, S. Jin, et al., The CRISPR-Cas toolbox and gene editing technologies, Mol. Cell 82 (2022) 333–347.
[148] A. Mayorga-Ramos, J. Zúñiga-Miranda, S.E. Carrera-Pacheco, et al., CRISPR-cas-based antimicrobials: Design, challenges, and bacterial mechanisms of resistance, ACS Infect. Dis. 9 (2023) 1283–1302.
[149] A. Zuberi, L. Misba, A.U. Khan, CRISPR interference (CRISPRi) inhibition of luxS gene expression in *E. coli*: An approach to inhibit biofilm, Front. Cell. Infect. Microbiol. 7 (2017), 214.
[150] M.F. Noirot-Gros, S. Forrester, G. Malato, et al., CRISPR interference to interrogate genes that control biofilm formation in *Pseudomonas fluorescens*, Sci. Rep. 9 (2019), 15954.
[151] T. Gong, B. Tang, X. Zhou, et al., Genome editing in *Streptococcus mutans* through self-targeting CRISPR arrays, Mol. Oral Microbiol. 33 (2018) 440–449.
[152] J.N. Hennigan, M.D. Lynch, The past, present, and future of enzyme-based therapies, Drug Discov. Today 27 (2022) 117–133.
[153] R. Ramakrishnan, A.K. Singh, S. Singh, et al., Enzymatic dispersion of biofilms: An emerging biocatalytic avenue to combat biofilm-mediated microbial infections, J. Biol. Chem. 298 (2022), 102352.
[154] X. Lv, L. Wang, A. Mei, et al., Recent nanotechnologies to overcome the bacterial biofilm matrix barriers, Small 19 (2023), e2206220.
[155] H. Dong, W. Xiu, L. Wan, et al., Biofilm microenvironment response nanoplatform synergistically degrades biofilm structure and relieves hypoxia for efficient sonodynamic therapy, Chem. Eng. J. 453 (2023), 139839.
[156] B. Wan, Y. Zhu, J. Tao, et al., Alginate lyase guided silver nanocomposites for eradicating *Pseudomonas aeruginosa* from lungs, ACS Appl. Mater. Interfaces 12 (2020) 9050–9061.
[157] J.L. Lister, A.R. Horswill, *Staphylococcus aureus* biofilms: Recent developments in biofilm dispersal, Front. Cell. Infect. Microbiol. 4 (2014), 178.
[158] S.K. Saggu, G. Jha, P.C. Mishra, Enzymatic degradation of biofilm by metalloprotease from *Microbacterium* sp. SKS10, Front. Bioeng. Biotechnol. 7 (2019), 192.
[159] D. Fleming, K.P. Rumbaugh, Approaches to dispersing medical biofilms, Microorganisms 5 (2017), 15.
[160] K. Sharma, A. Pagedar Singh, Antibiofilm effect of DNase against single and mixed species biofilm, Foods 7 (2018), 42.
[161] L. Karygianni, T. Attin, T. Thurnheer, Combined DNase and proteinase treatment interferes with composition and structural integrity of multispecies oral biofilms, J. Clin. Med. 9 (2020), 983.
[162] Y.H. Li, P.C. Lau, J.H. Lee, et al., Natural genetic transformation of *Streptococcus mutans* growing in biofilms, J. Bacteriol. 183 (2001) 897–908.
[163] P. Taslimi, İ. Gulçin, Antioxidant and anticholinergic properties of olivetol, J. Food Biochem. 42 (2018), e12516.
[164] K. Cetin Cakmak, İ. Gülçin, Anticholinergic and antioxidant activities of usnic acid-an activity-structure insight, Toxicol. Rep. 6 (2019) 1273–1280.
[165] İ. Gülçin, Antioxidant activity of food constituents: An overview, Arch. Toxicol. 86 (2012) 345–391.
[166] İ. Gulcin, Antioxidants and antioxidant methods: An updated overview, Arch. Toxicol. 94 (2020) 651–715.
[167] A.N. White, B.S. Learman, A.L. Brauer, et al., Catalase activity is critical for *Proteus mirabilis* biofilm development, extracellular polymeric substance composition, and dissemination during catheter-associated urinary tract infection, Infect. Immun. 89 (2021), e0017721.
[168] J. Wen, Z. Wang, X. Du, et al., Antibioflm effects of extracellular matrix degradative agents on the biofilm of different strains of multi-drug resistant *Corynebacterium striatum*, Ann. Clin. Microbiol. Antimicrob. 21 (2022), 53.
[169] Y. Li, R. Dong, L. Ma, et al., Combined anti-biofilm enzymes strengthen the eradicate effect of *Vibrio parahaemolyticus* biofilm: Mechanism on *cpsA-j* expression and application on different carriers, Foods 11 (2022), 1305.

[170] M. Ding, W. Zhao, X. Zhang, et al., Charge-switchable MOF nano complex for enhanced biofilm penetration and eradication, J. Hazard. Mater. 439 (2022), 129594.
[171] P.J. Weldrick, M.J. Hardman, V.N. Paunov, Enhanced clearing of wound-related pathogenic bacterial biofilms using protease-functionalized antibiotic nanocarriers, ACS Appl. Mater. Interfaces 11 (2019) 43902–43919.
[172] W. Hussain, X. Yang, M. Ullah, et al., Genetic engineering of bacteriophages: Key concepts, strategies, and applications, Biotechnol. Adv. 64 (2023), 108116.
[173] S. Liu, H. Lu, S. Zhang, et al., Phages against pathogenic bacterial biofilms and biofilm-based infections: A review, Pharmaceutics 14 (2022), 427.
[174] R. Abdulrahman Ashy, S. Agustí, Low host abundance and high temperature determine switching from lytic to lysogenic cycles in planktonic microbial communities in a tropical sea (red sea), Viruses 12 (2020), 761.
[175] Q. Wang, Z. Guan, K. Pei, et al., Structural basis of the arbitrium peptide–AimR communication system in the phage lysis–lysogeny decision, Nat. Microbiol. 3 (2018) 1266–1273.
[176] Y. Elahi, J. Nowroozi, R.M.N. Fard, Isolation and characterization of bacteriophages from wastewater sources on *Enterococcus spp.* isolated from clinical samples, Iran. J. Microbiol. 13 (2021) 671–677.
[177] Z. Chegini, A. Khoshbayan, M. Taati Moghadam, et al., Bacteriophage therapy against *Pseudomonas aeruginosa* biofilms: A review, Ann. Clin. Microbiol. Antimicrob. 19 (2020), 45.
[178] J.M. Loeffler, V.A. Fischetti, Synergistic lethal effect of a combination of phage lytic enzymes with different activities on penicillin-sensitive and-resistant *Streptococcus pneumoniae* strains, Antimicrob. Agents Chemother. 47 (2003) 375–377.
[179] K. Markoishvili, G. Tsitlanadze, R. Katsarava, et al., A novel sustained-release matrix based on biodegradable poly(ester amide)s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds, Int. J. Dermatol. 41 (2002) 453–458.
[180] G. Vukotic, M. Obradovic, K. Novovic, et al., Characterization, antibiofilm, and depolymerizing activity of two phages active on carbapenem-resistant *Acinetobacter baumannii*, Front. Med. 7 (2020), 426.
[181] O.D. Wiguna, D.E. Waturangi, Yogiara, Bacteriophage DW-EC with the capability to destruct and inhibit biofilm formed by several pathogenic bacteria, Sci. Rep. 12 (2022), 18539.
[182] T. Tkhilaishvili, L. Lombardi, A.B. Klatt, et al., Bacteriophage Sb-1 enhances antibiotic activity against biofilm, degrades exopolysaccharide matrix and targets persisters of *Staphylococcus aureus*, Int. J. Antimicrob. Agents 52 (2018) 842–853.
[183] S.C. Park, M.Y. Lee, J.Y. Kim, et al., Anti-biofilm effects of synthetic antimicrobial peptides against drug-resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus* planktonic cells and biofilm, Molecules 24 (2019), 4560.
[184] Y. Shao, C. Yin, F. Lv, et al., The *Sigma* factor AlgU regulates exopolysaccharide production and nitrogen-fixing biofilm formation by directly activating the transcription of *pslA* in *Pseudomonas stutzeri* A1501, Genes 13 (2022), 867.
[185] J.M. Dow, L. Crossman, K. Findlay, et al., Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants, Proc. Natl. Acad. Sci. USA 100 (2003) 10995–11000.
[186] B.R. Boles, M. Thoendel, P.K. Singh, Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms, Mol. Microbiol. 57 (2005) 1210–1223.
[187] G.A. Quinn, A.P. Maloy, M.M. Banat, et al., A comparison of effects of broad-spectrum antibiotics and biosurfactants on established bacterial biofilms, Curr. Microbiol. 67 (2013) 614–623.
[188] M.A. De Rienzo, P.J. Martin, Effect of mono and di-rhamnolipids on biofilms pre-formed by *Bacillus subtilis* BBK006, Curr. Microbiol. 73 (2016) 183–189.