

An Explorative Review on Advanced Approaches to Overcome Bacterial Resistance by Curbing Bacterial Biofilm Formation

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Abstract: The continuous emergence of multidrug-resistant pathogens evoked the development of innovative approaches targeting virulence factors unique to their pathogenic cascade. These approaches aimed to explore anti-virulence or anti-infective therapies. There are evident concerns regarding the bacterial ability to create a superstructure, the biofilm. Biofilm formation is a crucial virulence factor causing difficult-to-treat, localized, and systemic infections. The microenvironments of bacterial biofilm reduce the efficacy of antibiotics and evade the host's immunity. Producing a biofilm is not limited to a specific group of bacteria; however, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* biofilms are exemplary models. This review discusses biofilm formation as a virulence factor and the link to antimicrobial resistance. In addition, it explores insights into innovative multi-targeted approaches and their physiological mechanisms to combat biofilms, including natural compounds, phages, antimicrobial photodynamic therapy (aPDT), CRISPR-Cas gene editing, and nano-mediated techniques.

Keywords: bacterial biofilms, natural compounds, phages, aPDT, CRISPR, nanotechnology

Introduction

Considering the physiological role of bacterial virulence factors when targeting bacteria using conventional antibiotics is promising. Bacterial pathogenesis depends on protein-protein intercommunication, which is surface-bound or secreted to interact with the host-specific molecules or defense system.¹ The virulence of microbes is key to enabling them to invade a host, surpass its immune system, and cause an infection. The microbial elements accomplishing this invasion are called "virulence factors".² The function of virulence factors is not to instigate the infection; however, it aids the bacteria in surviving hostile environments (ie, the host's body), resulting in cellular damage.³ This virulence intensifies the ability of the bacterial cell to establish an organized community called biofilm, 10–1000 times more potent than the sessile bacterial cell when treated with conventional antibiotics.⁴

Bacterial cells in the biofilm are contained in a self-produced polymeric extracellular matrix (ECM),² and its characteristics are summarized in Figure 1.^{7,8,10,11} The altered phenotypic features in biofilm-forming bacteria include decreasing the transcriptional gene expression and translating proteins essential for bacterial cell metabolism, resulting in reduced metabolic activity.^{4,5} Biofilm formation increases the genes' expressing qualities, including adherence, quorum-sensing systems, and competence.⁶ The secretion of the ECM layer follows successful adherence, which causes colony

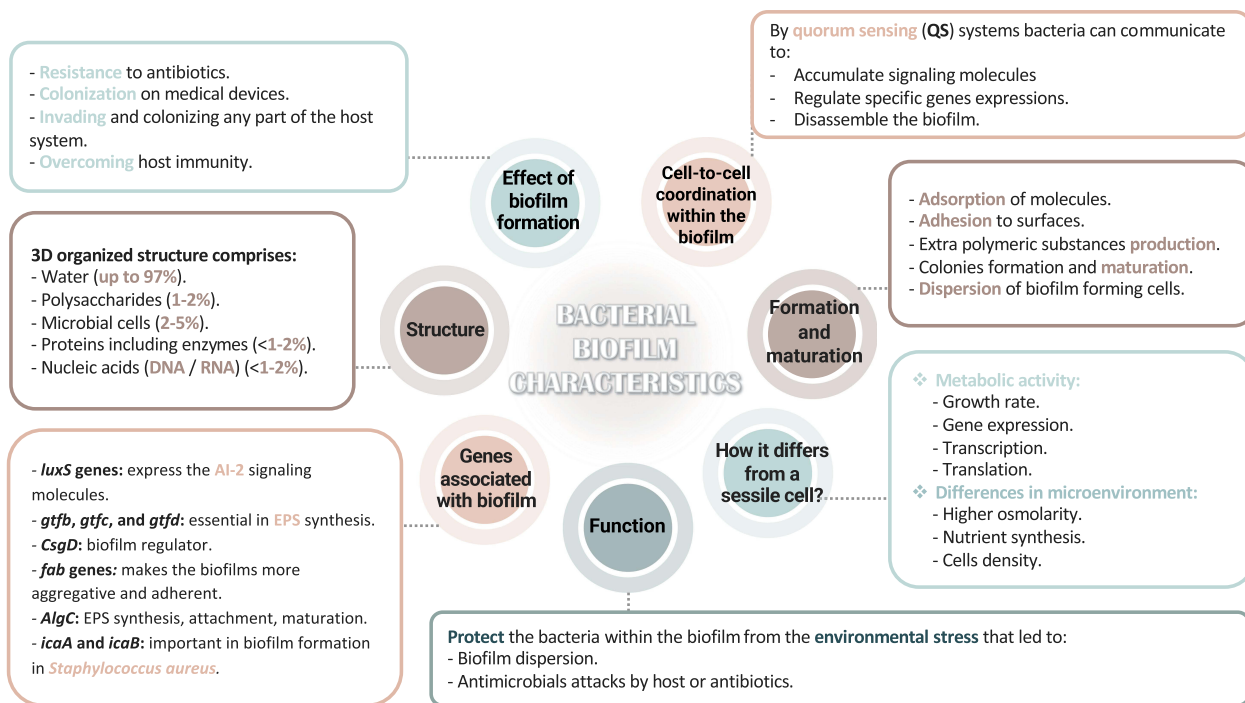


Figure 1 Bacterial biofilm characteristics.

Abbreviations: 3D, three-dimensional; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; *luxS* genes, quorum-sensing genes; *gtf(b,c,d)*, glycosyltransferases genes; *CsgD*, curli subunit gene; *fab*, fatty acid biosynthetic genes; *algC*, alginate encoding genes; *icaAB*, intracellular adhesion genes; AI-2, Auto-inducer 2; EPS, extracellular polymeric substances.

formation and maturation. The biofilm is dispersed to spread and localize to other sites.⁷⁻⁹ These interactions are controlled through a system known as quorum-sensing (QS), QS signaling molecules accumulate according to cell density.¹⁰ These molecules promote biofilm maturation and dispersion and regulate the expression of specific biofilm-related genes and proteins, such as *algC*, *fap*, *ica* operon, *gtfBCD*, and *CsgD*.^{7,11} Consequently, understanding the genetic and phenotypic interactions is essential to comprehend the development of biofilms during infection and to understand the crosstalk linking bacteria within the biofilm and their influence on antibiotic resistance.¹²

Targeting bacterial virulence includes targeting the bacterial attachment and invasion, biofilm formation, type 2 secretion system (toxin secretions), type 3 secretion system (protein transformation), response systems (two-component response systems; TCRs), and quorum-sensing systems.¹³ This approach is a specific method that differs according to the bacteria. It is a valuable tactic that can:

- (i) Promote the creation of new antibacterial agents with new targets.
- (ii) Decrease future resistance by avoiding pressuring the bacteria to evolve, due to selective pressure.¹⁴

This review summarizes and clarifies why biofilms are medically critical. First, we discuss numerous cited studies that exemplify how these clusters of bacteria grow in microbial communities due to forming a complex three-dimensional structure (biofilms). Secondly, we discussed the functions associated with the pathophysiology of biofilm-associated virulence factors in bacteria causing biofilm-associated infections. Lastly, we discuss innovative biofilm-eradication strategies using novel antibiofilm agents and approaches.

Biofilm-Forming Bacteria

The biofilm-forming bacteria can be 10 to 1000-fold more tolerant to antibiotics than sessile cells.¹⁵ The morphological changes in biofilm-forming bacteria promote surface adherence, hydrophobicity, low-level motility, and auto-

aggregation. Variations between sessile cells and biofilm-associated cells aid the survival of biofilms in hostile environments.^{5,16} Biofilm-associated infections are clinically challenging at many levels. They require multiple antibiotics to treat the disease, raising the likelihood of drug toxicity.¹⁷ However, combined antibiotic therapy causes antibiotic tolerance or resistance, resulting in persistent and difficult-to-treat infections.¹⁸ In addition, the lack of universal diagnostic protocols in Medical settings leads to delayed or improper diagnoses.¹⁹

Antibiotics tolerance is acquired due to two features in biofilm-forming bacteria:

- (i) The matrix formation alters the structural phenotype of the bacteria.
- (ii) The enlarged populations of bacterial persister cells (cells with decreased metabolism rate).⁶

As shown in Figure 2, the resistance mechanisms of antibiotics include efflux pumps (extruding the antibiotics to the bacterial periplasmic space), changing the expression of the membrane proteins (porins), decreasing the cellular membrane's permeability, and reducing small inhibitory molecules diffusion.^{20,21} These mechanisms might be similar in Gram-negative and Gram-positive bacteria, including overexpressing efflux pumps, inactivating/degrading antibiotics using enzymes (eg, β -lactamase), and mutating the targeted sites (yellow stars in Figure 2 highlight mutation sites). Other mechanisms differ according to the bacteria, such as blocking the permeability of antibiotics, covalently alternating antibiotics, overproducing PBPs, reducing antibiotic binding affinity, and protecting the targets of antibiotics using proteins.^{22–24}

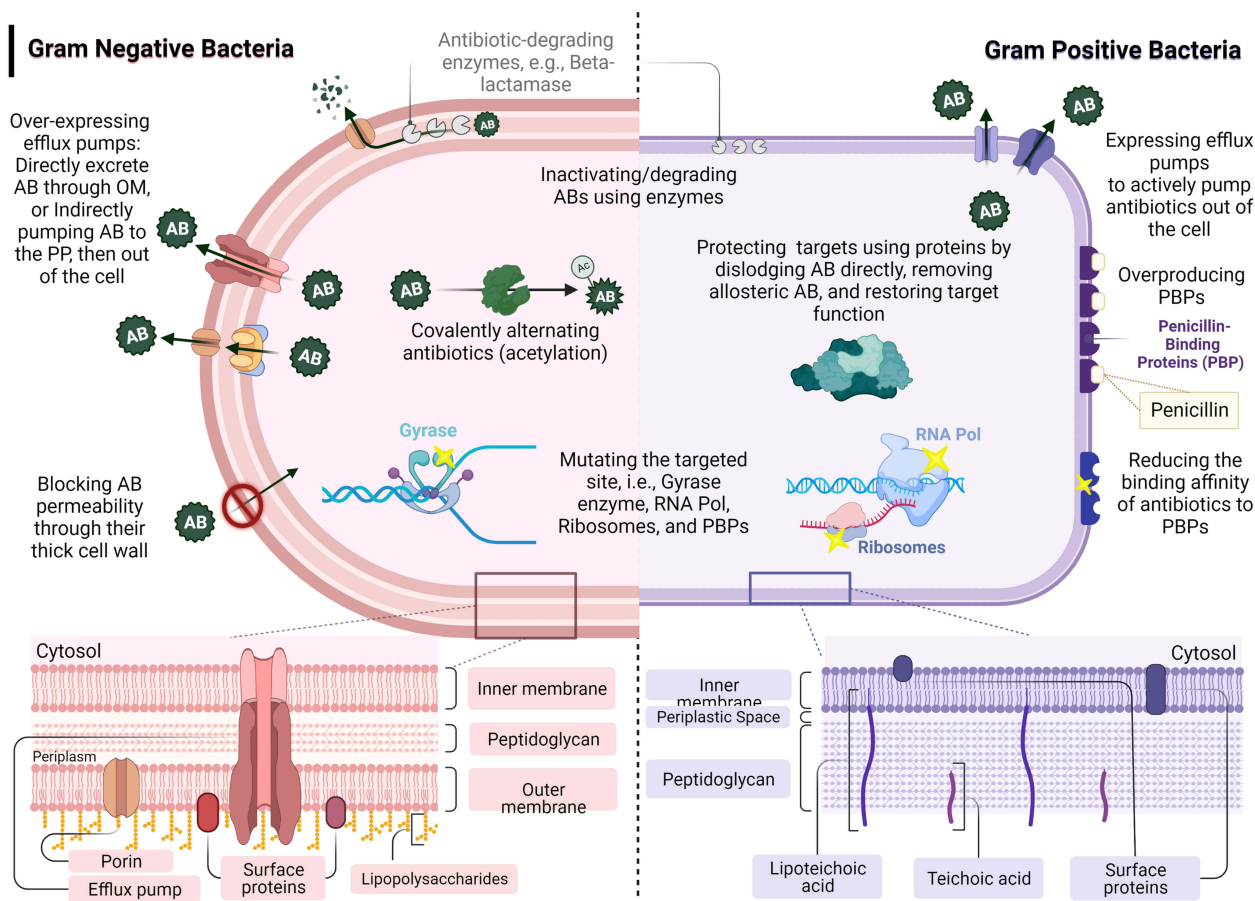


Figure 2 Bacterial biofilm resistance with different cellular mechanisms in Gram-positive and Gram-negative bacteria.

Notes: Data from these studies.^{22,25}

Abbreviations: AB/Abs, antibiotic; AC, acetylation; OM, outer membrane; PP, periplasm; PBPs, penicillin-binding proteins; RNA Pol, RNA polymerase.

Diagnosing biofilm-caused infections are dependent on laborious and expensive procedures. In addition, the antibiotic sensitivity assays measure the bacterial susceptibility in its planktonic form, not in the biofilm form, which may result in prescribing the wrong antibiotics or dosage.¹⁹ Researching antibiofilm drugs with specific, rapid, and cost-efficient diagnostic tools will advance the management of biofilm-caused infections.²⁶ Scientists are exploring simple and fast procedures to evaluate the susceptibility of bacterial biofilm and not solely depend on the susceptibility of planktonic cells.^{27,28}

Pseudomonas aeruginosa, *Acinetobacter baumannii*, and *Staphylococcus aureus* are known pathogens in the medical setting. Their ability to exert variable virulence factors, eg, biofilm formation, is essential during the disease they cause.^{29,30} We will discuss the current knowledge related to the role of biofilm formation in terms of their prevalence and medical significance.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is ubiquitous in diverse environments and notoriously resist antibiotics.³¹ Human infections caused by *P. aeruginosa* are prevalent; it is a primary opportunistic pathogen causing nosocomial infections using its hierarchical QS signaling pathways, regulates multiple virulence factors, and produces biofilm.³² Carbapenem-resistant Enterobacteriaceae *P. aeruginosa* (CRPA) is the leading cause of various healthcare-associated infections (HAI) in the United States, with more than 50,000 cases annually. This results from the presence of resistance plasmids with diverse virulence factors and metabolic pathways.³³

Pseudomonas aeruginosa is of the most prevailing Gram-negative biofilm-forming bacteria in medical settings.³⁴ It is responsible for 10%-20% of all HAI, which is challenging to treat if it colonizes the host tissue, specifically in cystic fibrosis (CF) patients. The biofilm matrix, known as the ECM layer, comprises of DNA, exopolysaccharides, and proteins. In addition, mucoid strains isolated from CF patients produce a plethora of exopolysaccharide alginic acid.³⁴ The proteinaceous constituents essential in biofilm formation are lectins- and carbohydrates-binding proteins. *P. aeruginosa* makes two lectins, LecA and LecB; both are important in biofilm formation and instigating infections.³⁵ Observing clinical *Pseudomonas aeruginosa* biofilm, versatile factors are expressed in response to environmental stressors and are activated to adapt to these stressors.³⁵ The essential elements in *P. aeruginosa* biofilm

Table 1 Summary of Required Components for Biofilm Formation and Maturation in *P. aeruginosa*

Factor	Identity or Chemistry	Responsible Gene/Operon	Function	Refs
Alginate	Exopolysaccharide/ O-acetylated 1–4 linked D-mannuronic acid and variable proportions of its five epimer L-guluronic acid	The alginate operon (<i>algD</i> , <i>alg8</i> , <i>alg44</i> , <i>algk</i> , <i>alagE</i> , <i>alagG</i> , <i>algX</i> , <i>algL</i> , <i>algI</i> , <i>algJ</i> , <i>algf</i> , and <i>alqA</i> and <i>alqC</i>)	Biofilm matrix formation in mucoid strains, Antibiotics tolerance and protection from host immunity, Aminoglycosides resistance	[32,38–40]
eDNA	Nucleic acid (extracellular DNA)	Cell Lysis	Virulence and biofilm matrix dynamics, Antibiotic tolerance, Nutrient source	[32,38,40,41]
Flagella	Multiprotein complex	At least 41 genes clustered in three regions of the genome encode flagellin structural and regulatory components	Irreversible adhesion and biofilm development	[32,42]

(Continued)

Table 1 (Continued).

Factor	Identity or Chemistry	Responsible Gene/Operon	Function	Refs
Pel	Exopolysaccharide/ partially acetylated (1–4) glycosidic linkage of <i>N</i> -acetylgalactosamine and <i>N</i> -acetylglucosamine	The <i>pelA-G</i> operon GDP-mannuronic acid	Biofilm formation on solid surfaces Biofilm matrix development and maintenance Virulence Aminoglycosides resistance	[32,38,40,43]
Psl	Exopolysaccharide/Repeating pentasaccharides containing D-mannose, D-glucose, and L-rhamnose	The <i>pslA-O</i> operon	Biofilm matrix formation, Antibiotic protection	[32,38]
Type 4 pili	Multiprotein complex/ Type 4a Pill	The <i>pilM/N/P/Q</i> and the <i>fimU-pilVWXYZIY2E</i> operons	Early attachment, adhesion, and biofilm development	[32,42]

formation and their genetics are listed in Table 1. These elements include extracellular DNA, exopolysaccharide (ie, alginate, Pel, Psl), and motility apparatus such as flagella and pili. Lipopolysaccharides (LPS) are potent constituents of *Pseudomonas* biofilm, comprising mainly O antigen (O-polysaccharide), oligosaccharides, and Lipid.³⁶ The LPS layer affects the host's immunity by suppressing the host's defenses and inducing hyperinflammatory reactions. This contributes to the chronic nature of the biofilm-associated infections in *P. aeruginosa* by producing key virulence factors, ie, biofilm formation and toxin (Lipid A) secretion.^{36,37} Comprehending the dynamics underpinning biofilm formation in *P. aeruginosa* is vital to develop appropriate antibiofilm agents, as these factors can be used as targets for new approaches.

Quorum-sensing expresses more than 300 genes, including genes fundamental in biofilm formation and the virulence in *P. aeruginosa*.^{44,45} The QS-dependent virulence factors produced for the pathophysiology of *P. aeruginosa* are listed in Table 2. In *Pseudomonas*, virulence factors and QS-molecules are synthesized differently during biofilm formation,

Table 2 Representation of QS-Dependent Virulence Factors in *P. aeruginosa*

Virulence Factor	Class or Chemistry	Encoding Gene	Secretion	Function	Refs
Alkaline Protease (aeruginolysin)	Extracellular enzymes-aided invasion/ M10 peptidase family/ zinc-metalloendopeptidase	<i>aprA</i>	Type 1 secretion system (T1SS)	Wide protease activity, tissue damage, immune system evasion, iron accessibility	[32,47]
Exotoxin A	Toxin/ PE belongs to the two-component AB toxin family/ NAD ⁺ - diphthamide-ADP-ribosyltransferase	<i>toxA</i>	Type 2 secretion system (T2SS)	Toxicity, immunosuppression, modifying the elongation factor 2 in eukaryotic cells, tissue damage, and death	[32,47]
Hydrogen cyanide (HCN)	Toxic secondary metabolite	<i>hcnABC</i> operon	Diffusible	Highly toxic/potent inhibitor of cytochrome c oxidase and other metalloenzymes, cytotoxicity, colonization	[32,47]
LasB Elastase	Extracellular enzymes-aided invasion/ M4 thermolysin peptidase family/ zinc metalloprotease	<i>lasA</i>	The Sec pathway and Type 2 secretion system (T2SS)	Protease and elastolytic activity, cleaving a wide range of glycine-containing proteins, tissue damage, immune system evasion, and invasion	[32,47]

(Continued)

Table 2 (Continued).

Virulence Factor	Class or Chemistry	Encoding Gene	Secretion	Function	Refs
LasB Elastase	Extracellular enzymes-aided invasion/ B-lytic zinc metalloendopeptidase (staphylolytic)/ serine protease	<i>lasB</i>	The Sec pathway and Type 2 secretion system (T2SS)	Protease and elastolytic activity, invasion, infection, tissue damage, iron accessibility	[32,47]
Lectin A	Toxic secondary metabolite/ Tetrameric protein	<i>lecA</i> (or <i>pal L</i>)	Intracellular; only a small fraction present on the cell surface	Galactophilic, adhesive, colonization, infection	[32,47]
PilC	Extracellular enzymes-aided invasion/ Phospholipases C	<i>plc B</i>	The Sec pathway and Type 2 secretion system (T2SS)	Hydrolyzing phosphatidylcholine and phosphatidylethanolamine, invasion, programmed tissue death, iron accessibility	[32,47]
Pyocyanin	Toxic secondary metabolite/ tricyclic phenazine	<i>phzA1-G1</i> and <i>phzA2-G2</i>	Type 2 secretion system (T2SS)	Redox-active, immunity evasion, colonization, toxicity	[32,47]
Pyoverdine	Iron acquisition/ Pyoverdines/ dihydroquinoline-type chromophore linked to a peptide	Large multi-modular enzymes/ non-ribosomal peptide synthetases (NRPSs)	PvdRT-opmQ Efflux pump and MexAB-OprM efflux pumps	High affinity to Fe (III) /iron acquisition/ fluorescent, tissue invasion, biofilm development	[32,47]
Rhamnolipids	Toxic secondary metabolite/ Rhamnose-containing glycolipid compounds	<i>rhlAB operon and rhlC</i>	-	Biosurfactant, detergent, structure, hemolytic activity, biofilm dispersal, toxicity	[32,47]

adhesion, formation, maturation, and dispersion. This contributes to surface attachment, stability, nutrition, cellular arrangement, and protection against antibiotics and host immunity.⁴⁶

Earlier studies noted the effect of biofilm formation by *P. aeruginosa* on infection and antibiotic resistance.^{40,48–50} A prospective study that monitored the HAI incidence in Rome for 24 months, reported a prevalence of *Pseudomonas* infections of 25%.⁵¹ A meta-analysis of studies between 2000 and 2010 reported *P. aeruginosa* as the most frequently found wound pathogen.⁵² This prevalence urged Researchers to study the effectiveness of new approaches to combat this pathogen. For instance, anti-virulence drugs proved effective by disarming the bacteria rather than targeting their replication and growth. As a result, the infection rate will eventually slow down, allowing the immune system to attack the invading pathogen.⁵³ A recent study by Rezzoagli and researchers demonstrated a novel procedure by combining antibiotics and anti-virulence drugs, Gallium (siderophore-binding molecule) and furanone C-30 (QS-inhibitor), to treat *P. aeruginosa* in vitro.⁵⁴ Their approach proved effective against the bacteria in a concentration-dependent manner. They observed a promising synergy at intermediate drug concentrations for specific drug pairs.⁵⁴

Acinetobacter baumannii

Acinetobacter baumannii represents a unique species. It is possible to isolate *A. baumannii* from Healthcare facilities, food, water, and soil. Literature highlighted their clinical significance due to the increase in carbapenem-resistant strains in hospitals and communities.⁵⁵ Infections caused by *A. baumannii* are increasingly challenging for healthcare professionals globally, particularly in intensive care units. The multi-drug resistance (MDR) rate has increased significantly in recent years, with a tolerance to last-line antibiotics, such as colistin.⁵⁶

The World Health Organization (WHO) labelled *A. baumannii* a “critical” pathogen, constituting a significant global human health risk.⁵⁷ This opportunistic pathogen mediates pathophysiology mechanisms, including bacterial virulence factors (eg, biofilm formation), genes (eg, resistance), and host immune responses (eg, invasion)⁵⁵ (Table 3). Biofilm

Table 3 Summary of Virulence Factors, Genes, and Pathogenesis of *A. baumannii*

Virulence Factors or Genes	Role in Pathogenesis	Model	Refs
Porin (<i>OmpA</i>, <i>Omp33-36</i>, <i>Omp22</i>, <i>CarO</i>, <i>OprD</i>-like)	Adherence and invasion, Induction of apoptosis, serum resistance, Biofilm formation, persistence	In vitro studies and induced tracheal aspiration pneumonia by AB 5075 strain in C57BL/6 mice; Growth rate and cell cytotoxicity of clinical isolate compared to ATCC 19606; intraperitoneally challenged C57BL/6 mice with <i>Omp33-36</i> knockout in AB 17978 compared to wild type	[59–71]
Capsular polysaccharide	Growth in serum, survival in tissue infection, biofilm formation	C57BL/6 mice intraperitoneally challenged with stimulated AB ATCC 17978 to form capsule compared to capsule-mutated strains	[59,60,72–75]
Lipopolysaccharide (LPS) genes	Serum resistance, survival in tissue infection, evasion of the host immune response	BALB/c mice intraperitoneally challenged with knockouts in AB ATCC 19606 compared to wild types.	[59,60,76–81]
Phospholipase (PLC and PLD) LipA (lipase)	Serum resistance, invasion, in vivo survival	Neutropenic tail vein infection of DBA mice infected with LipA knockout in <i>A. baumannii</i> ATCC 17978 and compared to wild type	[59,60,82–86]
Outer membrane vesicle (OMV)	Delivery of virulence factors, horizontal transfer of antibiotic resistance gene	<i>A. nosocomialis</i> ATCC 17903 purified from OMVs and administered in vitro to cells and BALB/c mice model	[59,60,87–93]
Iron acquisition system – siderophore (Acinetobactin and <i>NfuA</i>)	In vivo survival, persistence, the killing of host cells	Galleria and C57BL/6 mice models with intraperitoneally infected with acinetobactin knockouts AB ATCC 19606 and compared to the wild type	[59,60,85,94–99]
Zinc acquisition system (ZnuABC and <i>ZigA</i>)	In vivo survival	C57BL/6 mice challenged with intranasal infection by <i>A. baumannii</i> ATCC 17978 or a <i>ZigA</i> knockout strain	[59,60,94,95]
Manganese acquisition system (MumC and MumT)	In vivo survival	—	[59,100]
Type 2 protein secretion system (T2SS); <i>gspD</i>	In vivo survival and an intermediate effect on bacterial population density	C57BL/6 mice received nasal inoculation of a <i>gspD</i> -knockout strain of <i>A. nosocomialis</i> M2 and compared to the wild type	[59,60,86,101,102]
Type 6 protein secretion system (T6SS)	The killing of competing bacteria, host colonization	T6SS was analyzed in AB ATCC 17978, AB DSM30011 non-clinical isolates, and three clinical isolates	[59,60,103–106]
Type 5 protein secretion system (T5SS)	Biofilm Formation, adherence	—	[59,107]
Penicillin-binding protein 7/8/ and β-Lactamase	Serum resistance, in vivo survival, adherence	—	[59,108,109]
<i>PER-1</i>			
<i>CipA</i>	Serum resistance, invasion	—	[59,110]
<i>Tuf</i>	Serum resistance	—	[59,111]
<i>RecA</i>	In vivo survival and repairing damaged DNA	CD1 mice with intraperitoneal infection using <i>A. baumannii</i> ATCC 17978 after <i>RecA</i> knockout compared to wild type	[59,60,112]
<i>SurA1</i>	Serum resistance, in vivo survival, growth rate	Chicks infected with Knockout of <i>SurA1</i> <i>A. baumannii</i> CCGGD201101	[59,60,113,114]
GigABCD	In vivo survival, the killing of host cells	—	[59]
Universal stress protein A (UspA)	In vivo survival, the killing of host cells, pathogenesis of sepsis and pneumonia infections	C57BL/6 mice were challenged intranasally and intraperitoneally using the <i>UspA</i> knockout strain of AB ATCC 17978 and compared to the wild type	[59,60,113,115]

(Continued)

Table 3 (Continued).

Virulence Factors or Genes	Role in Pathogenesis	Model	Refs
Sensor kinas (GacS) and catabolic pathway of phenylacetic acid (PaaE)	Neutrophil influx	DBA mice challenged with intraperitoneal infection of knockout in AB ATCC 17978 and compared to wild type	[59,60,113,116,117]
Pili	Adherence, biofilm formation	—	[59,118,119]
OmpR/EnvZ	The killing of host cells	—	[59,120]
FepA (enterobactin receptor)	Competitive growth rates	CBA/J mice challenged with intravenous infection by <i>A. baumannii</i> ATCC 17978 with a mutant transposon library	[59,60,121]
AbeD	The killing of host cells	—	[59,122]
gacA and gacS (regulator genes)	—	Zebrafish embryos challenged with AB ATCC 17978 and knockouts	[60,117]
Biofilm gene (LH92_11085)	Biofilm formation	Identification of gene expression level and biofilm formation in <i>A. baumannii</i> MAR002	[60,123]

formation is a characteristic that results in the survival of *A. baumannii* in the presence of antibiotics and stressors. *A. baumannii* causing wound infections produces virulence factors that lengthen the treatment course, especially in burn patients, elevating the mortality and morbidity rates.⁵⁸

In a systematic review and meta-analysis, scientists investigated the association between the ability to form biofilm in *A. baumannii* in patients suffering from burns, and the antibiotic resistance trend. Results indicate that more than 90% of *A. baumannii* strains produced biofilms, contributing to antibiotic resistance in the burn units.⁵⁸

Altınok and others compared virulence genes and *A. baumannii*'s ability to form a biofilm and revealed that the biofilm formation was mostly related to genes encoding curli fiber (*csgA*), the chaperone-usher fimbria (*csuE*), and the outer membrane (*ompA*).¹²⁴ Different virulence genes such as *csgA*, *csuE*, *fimH*, *ompA*, and *blaPER-1* were investigated, and it was determined that 60.3% of the isolates formed biofilm. The frequency of *csgA*, *csuE*, *ompA*, *fimH* and *blaPER-1* genes of all isolates were 71.2%, 32.1%, 21.8%, 7.1%, and 3.2% respectively. The frequency of *csgA*, *ompA*, *bap*, *csuE*, and *fimH* virulence genes of the biofilm-producing isolates was 41.5%, 24.5%, 20.2%, and 5.3%, respectively. All the genes studied were abundant in the isolates with a strong and medium-positive biofilm production. This demonstrates that, except for the *fimH* gene, the *csgA*, *csuE*, and *ompA* genes contributed to the biofilm formation in invasive *A. baumannii* isolates.¹²⁴

Another review by Colquhoun and Rather investigated the genetic regulation mechanism of biofilm formation in *A. baumannii*.¹²⁵ The research divided the known 132 up-regulated biofilm genes in the outer membrane proteins, attachment/motility, metabolism, transcription, translation, and hypothetical proteins. The top categories were metabolism (49 genes), translation (30 genes), and outer membrane proteins (29 genes). This indicates that the transition and maintenance of the biofilm environment require alterations in the metabolic pathways and configuration of the outer membrane, assisted mainly through the translation machinery required for the production of nascent proteins.¹²⁵ The seriousness of *A. baumannii* infections is due to its resistance to the latest line of antibiotics, colistin, which mandates researching new treatment approaches. Srisakul and others reported novel synergistic activity between colistin and sulbactam against clinical isolates of colistin-resistant *A. baumannii*. This combination was tested in mouse models and in vitro; combinatorial therapy may provide a new option to treat this critically alarming pathogen.¹²⁶

Staphylococcus aureus

Staphylococcus aureus is of the most ubiquitous and dangerous facultative intracellular human pathogens due to its virulence and ability to develop antibiotic resistance.¹²⁷ Methicillin-resistant *Staphylococcus aureus* (MRSA) transmission occurs from infected patients, healthcare personnel, or items colonized with MRSA due to contact, known as

hospital and community transmission of MRSA, respectively.¹²⁸ *S. aureus* produces extracellular enzymes important for the pathophysiological mechanisms and regulates novel virulence factors. Multiple environmental factors, including nutrients, antibacterial agents, pH, shearing force, and temperature influence the stages of biofilm formation. These stages are attachment, maturation, detachment, and development.¹²⁹ They significantly influence several virulence determinants that evoke the host's immune response to the bacteria. Biofilm-associated infections are correlated with intravascular catheters or attachment to medical implants and host tissue.¹²⁹ Some Staphylococcal diseases are toxin-mediated because many strains produce exotoxins, such as toxic shock syndrome (TSS toxin 1), scalded skin syndrome (exfoliative toxin), and food poisoning (enterotoxin).¹³⁰ Surface and secreted proteins are essential for the mechanisms of pathophysiology and regulation of novel virulence factors for *S. aureus* (Figure 3).

One of the features of *S. aureus* biofilm infections is its ability to survive on biotic and abiotic surfaces and its tendency to resist drugs.¹³² If we compare MRSA infections with all other infections caused by MDR Gram-negative bacteria, it is 10 times more prevalent. The WHO included MRSA as one of the 12 urgent pathogens jeopardizing human health.¹³² Notably, the genotypic variation of *S. aureus* strains may play a role in biofilm production, but evidence is still insufficient to support these associations.¹³³

S. aureus formulate four types of biofilms: polysaccharide biofilm, protein-mediated biofilm, fibrin biofilm, and amyloid biofilm.¹³⁴ The polysaccharide intercellular adhesin (Pia) protein, involved in intercellular adhesion and biofilm formation, is a documented virulence factor in the *S. aureus* cell wall.¹³⁵ This element is also the first identified mediator of biofilm formation in *Staphylococcus epidermidis*, which has formed the foundation of other biofilm mechanisms in *S. epidermidis* and *S. aureus*.¹³⁴ Studies indicate that *S. aureus* produces biofilm using proteins instead of polysaccharides (protein-mediated biofilms) in an *ica*-

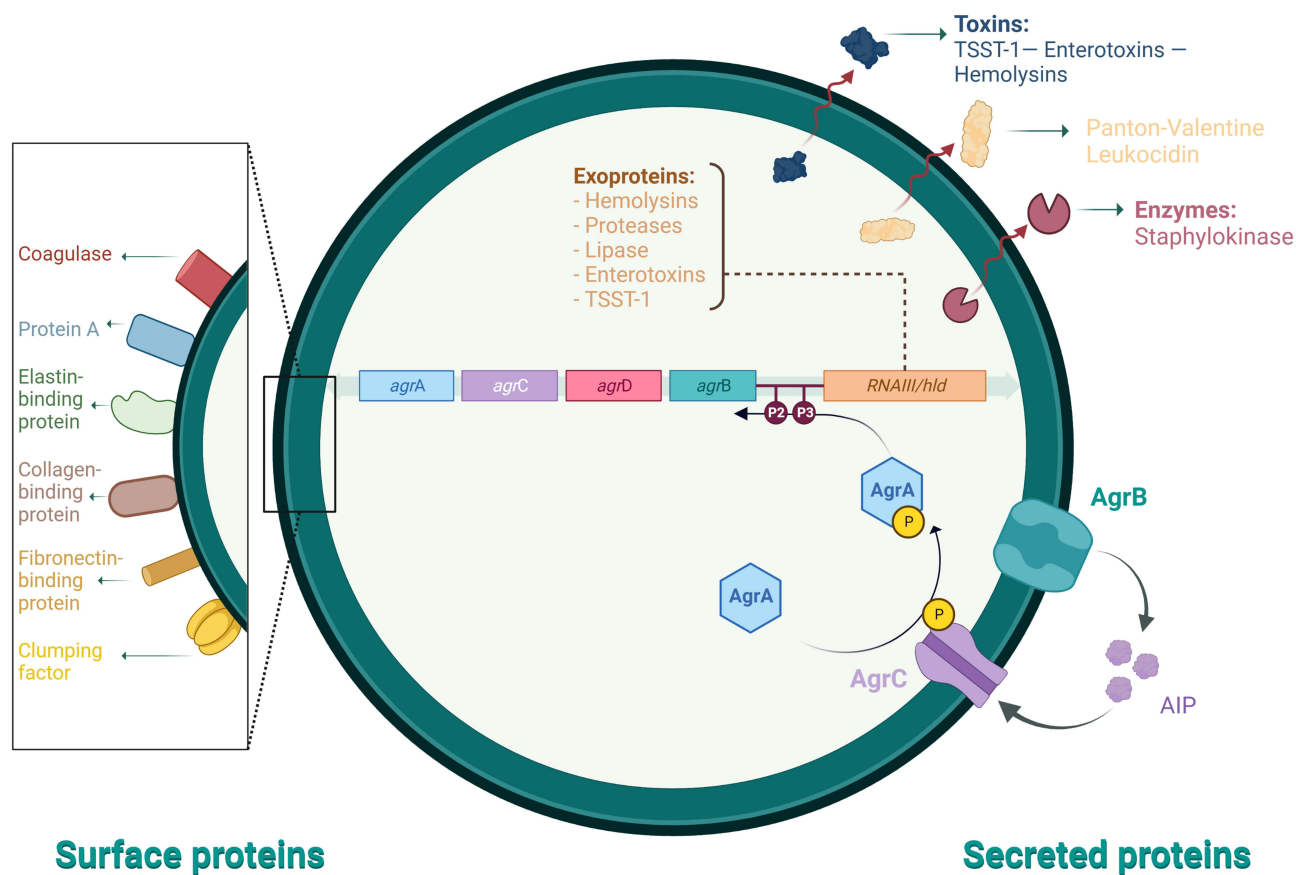


Figure 3 *Staphylococcus aureus* pathophysiology mechanisms and regulation of virulence determinants.

Notes: Data from these studies.^{129,131}

Abbreviations: TSST-1, toxic shock syndrome toxin 1; P1 and P2, promoters; *agrABCD*, accessory gene regulator genes operon and the precursors for AgrABC proteins; RNAIII, RNAIII transcript yields RNA as the primary effector; AIP, autoinducing peptide.

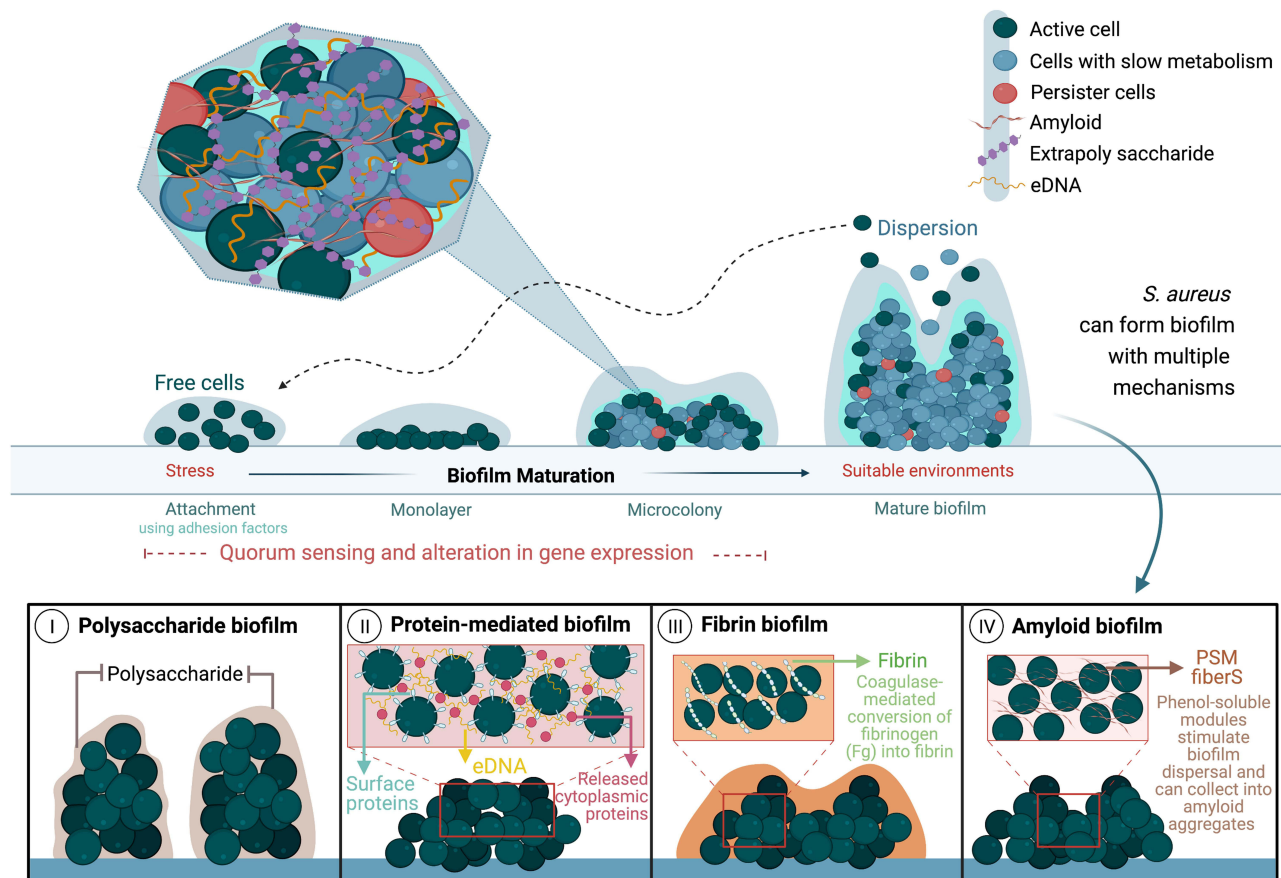


Figure 4 Stages of *S. aureus* biofilm production and its different formation mechanisms.

Notes: Data from these studies.^{134,140}

Abbreviations: eDNA, extracellular DNA; Fg, fibrinogen, PSM fibers, phenol-soluble modulins fibers.

independent manner¹³⁶ (Figure 4). Numerous surface adhesins are involved in protein-mediated biofilm production, including Bap, Spa, FnBPA, FnBPB, and SasG. These proteins are referred to as “cell wall-anchored proteins.” They enable *S. aureus* to resist polysaccharide-degrading enzymes, such as dispersin B, and survive harsh environments.^{137–139} Fibrin biofilm forms mostly during blood infection when staphylococcal coagulase attaches to the host’s prothrombin forming a complex, which causes the conversion of fibrinogen in the blood to fibrin, shielding the biofilm from its surroundings.¹³⁴ Phenol-soluble modulins produce amyloid biofilm after accumulating in the amyloid fibers that improve biofilm formation (Figure 4).¹³⁴

Biofilm formation impacts the patient experience by affecting their chances of being readmitted within 90 days of discharge or decreasing their 90-day mortality rate.¹³³ Luther et al, 2018, studied the connection between strong and weak biofilm formation in *S. aureus* and its ramification on hospitalized patients. Results showed links between the strong biofilm production in MRSA and *agr* genes deficiency, pigmentation, and administration of immunosuppressants or chemotherapy within the last 90 days.¹³³ Research related to the effect of the genotypic variations in *S. aureus* strains, including MRSA, and its magnitude on biofilm formation is urgently required.

To summarize, many bacteria can produce a structured ecosystem called the “biofilm.” Gram-positive and -negative bacteria possess varying morphology, genetics, and physiologies, but several biofilm-formation dynamics are comparable between the two types of bacteria.¹⁴¹ However, researchers suggest exploring how these dynamics might change in polymicrobial biofilms, comprising the two types of bacteria or other species, and their contribution during infection. *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* are associated with most human infections and known for their inherent tolerance to antimicrobials and the host’s immune responses.^{142–144} These medically prevailing pathogens pose a global burden, primarily in hospitals and communal areas, that adversely influence the patient experience and are costly to treat. Biofilm-associated infections challenge the current therapeutics and diagnostics. More surveys of the

incidence of infection must be done to provide evidence for the public and scientific community to encourage a hygienic lifestyle and urge researchers to investigate novel control approaches. Table 4 summarizes medically important Gram-positive and negative biofilm-forming bacteria, associated infections, and numerous innovative research and treatment approaches.

Table 4 Biofilm-Associated Infections and Innovative Treatment Approach

Bacteria	Biofilm Infection	Approach	Refs
Gram-negative, obligate Aerobic			
<i>Escherichia coli</i>	Urinary tract infections (UTI) Neonatal sepsis Meningitis Catheter-associated urinary tract infections (CAUTI) Crohn's disease and enteric syndromes associated infections Enteric hemorrhage Veterinary infectious diseases, including mastitis	Anti-adhesion agents Phage therapy Antimicrobial peptides Natural compounds Nanocarriers Combinatory therapy, ie, CRI-dots nanocomplex (CRISPR-Cas and Carbon quantum dots)	[145,146]
<i>Pseudomonas aeruginosa</i>	Nosocomial infection Chronic infections in immunocompromised patients Chronic lung infection Chronic wound infection Chronic rhinosinusitis	Anti-biofilm strategies (eg, Fimbriae or pili assembly blockers, OmpA inhibitors) Antiquorum-sensing (QS) molecules (eg, AHL, abaR receptors) Extracellular polymeric substances (EPS) inhibitors Antibacterial agents (iron-chelators) Antimicrobial peptides Advanced nanocarriers	[46,147]
<i>Acinetobacter baumannii</i>	Nosocomial infections Ventilator-associated pneumonia Urinary tract infections Wound infections Bacteremia Endocarditis Meningitis Severe community-associated infections	Antibiofilm peptides, inhibitors of natural and synthetic sources, anti-QS molecules, degrading enzymes, combinatorial therapy of essential oils and nanocarriers (eg, polymer, metal, and silica-based nanoparticles) Phage therapy Photodynamic therapy	[148–151]
<i>Salmonella sp, S. Enterica serovar Typhi, S. typhimurium</i>	Typhoid fever bowel perforation septicemia; meningitis; Catheters-associated infections Non-typhoidal salmonellae diseases, ie, chronic gallbladder infection Recalcitrant typhoid fever leading to hepatobiliary cancers	EPS production inhibitors Antimicrobial Peptides CurlI amyloid fibers inhibitor by a human monoclonal antibody Combinatory therapy, ie, ultrasound and disinfectants, halogenated furanones and antibiotics or disinfectants, and nano- and micro-emulsion	[152–157]
<i>Klebsiella pneumonia</i>	Community-acquired and nosocomial infections Medical devices-associated infections Urinary and biliary tract infections Osteomyelitis Bacteremia Chronic endometritis-associated infections Mastitis Prostatitis	Natural compounds (eg, 3-methyl-2(5H)-furanone and 2'-hydroxycinnamic acid) Phage therapy Biofilm matrix-degrading enzymes Histidine Functionalized Silver Nanoparticles Combinatory therapy, ie, non-ionic surfactants and phages; carboxypterin, methylene blue, and antimicrobial photodynamic therapy (aPDT); nanotechnology aPDT, and antimicrobial photothermal therapy (aPTT).	[152,158–164]

(Continued)

Table 4 (Continued).

Bacteria	Biofilm Infection	Approach	Refs
Gram-positive, facultative Anaerobic			
<i>Staphylococcus aureus</i>	Mastitis Chronic Endometritis-associated infections Laryngitis Pharyngitis Catheters-associated infections (eg, intravenous catheters, urinary catheters, dialysis catheters) Infections associated with implanted medical devices (eg, pacemakers, joint prostheses, and fluid shunts)	Antibiofilm surfaces: Anti-adhesion agents (eg, polyethylene glycol (PEG) coating) Antifoulants (coatings using paints containing metal nanoparticles) aPDT Laser Shock waves (LSW) Antimicrobial peptides Enzymes Phage therapy Combinatory therapy, ie, ultrasound and disinfectants, using multiple antibiotics.	[152,154,165,166]
<i>S. epidermidis</i>	Medically implanted devices infections Skin and soft tissue infections Bone and muscle infections Lung infections Bloodstream infections	QS-inhibitors (eg, thiophenones and furanones) EPS dispersion agents (eg, β -glucosidase, N-acetylcysteine; NAC) Antibiofilm surfaces: Anti-adhesion agents (PEG coating) Antifoulants (metal nanoparticles-containing paints) Combinatory therapy, eg, nanotechnology and aPDT, antimicrobial photothermal therapy (aPTT)	[157,159,161–163,165,167,168]
<i>Streptococcus pneumoniae</i>	Colonization of the nasopharynx leads to otitis media Chronic Endometritis associated infections Rhinosinusitis Laryngitis Pharyngitis	Nanotechnology (eg, Zinc oxide nanoparticles) Inhibition by small molecules (eg, Quercetin, DNA adenine methyltransferase Inhibitor, and Pyrimidinedione)	[152,169–171]

Innovative Approaches to Curb Biofilm Formation

Biofilm is present in more than 90% of bacteria and impacts bacterial infection significantly.¹⁷² The increasing incidence of multi-drug-resistant bacteria warrant the development of novel interventions. Bacterial virulence management is an interesting viewpoint compared to antibiotic therapy, which provides methods to prevent pathogenesis without adding stress on the targeted bacteria.^{13,173} This review explores innovative techniques that might affect the control and defense against biofilm formation by targeting bacterial virulence factors. These approaches include phytochemicals, antimicrobial photodynamic therapy (aPDT), bacteriophages, gene editing by CRISPR-CAS, and nano-mediated techniques.

Natural Products (Phytochemicals)

New methods widely explored phytochemicals extracted from natural sources to prevent biofilm formation and quorum-sensing.^{174,175} Phytochemicals, alone or in combination, have been used to repurpose old antibiotics or reduce the dose of antibiotics.^{176,177} Human cultures have been using herbal remedies for centuries, and some of these natural products are useful in treatment and prevention.¹⁷² Traditional Chinese medicinal herbs, for instance, were widely used in treating bacterial infections and prevention, and their antibacterial capacity was demonstrated in some herbs, such as Scutellaria, Taraxacum, and Tussilago. Plant extracts have also recently been described to control biofilm development and inhibit quorum-sensing (QS) in bacteria.¹⁷²

Many natural anti-biofilm compounds, such as phytochemicals, biosurfactants, and antimicrobial peptides, are effective against a broad range of microbial biofilms with different modes of action.¹⁷⁸ Novel natural antibiofilm agents such as phytochemicals broadly comprise numerous natural compounds with anti-biofilm properties: phenolics,

terpenoids, lectins, alkaloids, polypeptides, and polyacetylenes. These natural phytochemicals, with their mechanisms of action (Figure 5), can regulate QS and quorum-quenching (QQ) systems, crucial players in bacterial biofilm formation, virulence factors and antibiotic production in Gram-negative and positive bacterial infections.¹⁷⁸

The bacterial QS systems received increasing research focus. Targeting the bacterial QS system is an efficient strategy to prevent biofilm formation. Evidence shows that QS inhibitors obtained from natural compounds, such as 1,2-benzenedicarboxylic acid, diisooctyl ester play a significant role in inhibiting bacterial adhesion and suppressing biofilm-related genes.¹⁷⁹ Singh and others suggested that phytochemicals down-regulate biofilm formation genes by competing with the QS-molecules, such as *N*-Acyl homoserine lactones (AHL) and autoinducers (AIs) and preventing their binding to these genes.¹⁷⁹ Scientists are exploring the therapeutic value of traditional medical practices by using natural products and their effect on QS systems. Research focuses on studying natural QS-controlling substances and their mechanisms, to treat microbial-related diseases and impede antibiotic resistance.^{180–182} Literature suggests using green methods to manage biofilm, including enzymes, phages, and bioregulation in the food industry.^{183,184} Using detergents from enzymes as bio-cleaners, also called green chemicals, will support solving the issue of biofilm in the dietary industry.¹⁸⁵

Reen et al reviewed the potentiality of using coumarins to inhabit biofilm and QS. Coumarins are a vast family of naturally derived fused benzene and α -pyrone rings. They are predominantly present in plants with many pharmacological properties. Examples of coumarin compounds with antibiofilm activity include ellagic acid, warfarin, nodakenetin, and fraxin.¹⁸⁰ Girennavar and others explored the anti-biofilm and anti-QS ability of a constituent containing joined coumarins and furan molecules known as furocoumarins.¹⁸⁶ This constituent (extracted from grapefruit) showed similar activity to coumarins and inhibited 95% of the autoinducer-1 (AI-1) and autoinducer-2 (AI-2) signaling systems in the biosensor strain *Vibrio harveyi*. It inhibited biofilm formation in Gram-negative bacteria, ie, *Escherichia coli*, *Salmonella typhimurium*, and *P. aeruginosa*.^{186,187}

Other researchers tested seven structurally related coumarin compounds against *P. aeruginosa* and *Chromobacterium violaceum* (biosensor strain).¹⁸⁸ The results indicated that these compounds, largely the compounds retaining a hydroxyl group, affect QS and biofilm formation in bacteria. This phytochemical impacted biofilm formation in *P. aeruginosa* but not bacterial growth, suggesting that this inhibitory action targeted the QS-signaling system in the bacteria.¹⁸⁸

Many natural compounds, medicinal plants and phytochemicals, contributed to the research and discovery of antibiofilm agents. For example, *Allium subhirsutum* L. (hairy garlic) proved to have antibiofilm, anti-quorum-sensing, and antibacterial activity against different bacterial and fungal isolates.¹⁸⁹ *Eruca sativa* Miller was tested against numerous food-borne pathogens for their antibacterial and antibiofilm activity and demonstrated reduced bacterial

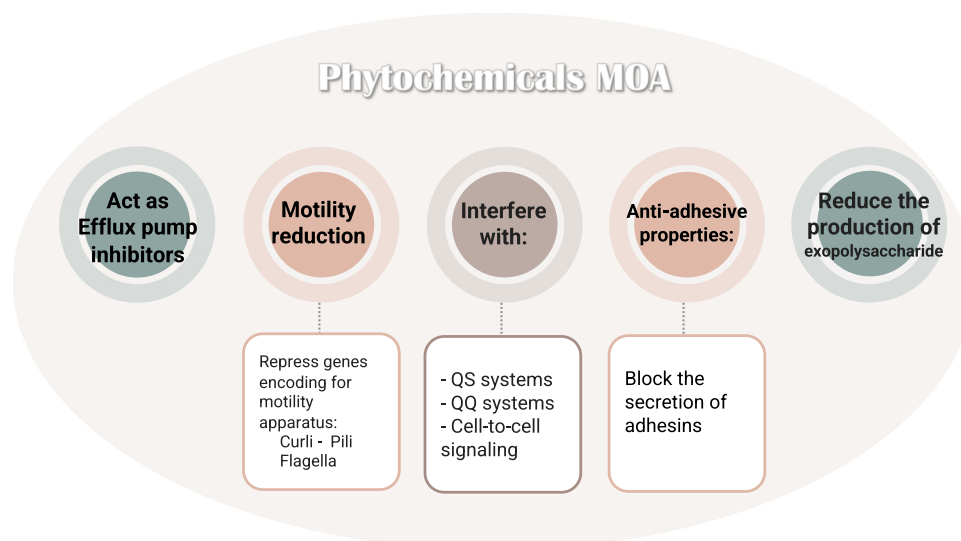


Figure 5 Presents the numerous modes of action of phytochemicals on various biofilms.

Notes: Data from Mishra et al.¹⁷⁸

Abbreviations: QQ, quorum-quenching; QS, quorum-sensing.

density within the biofilm community and a disturbed integrity of the biofilms by interfering with exopolysaccharides synthesis.¹⁹⁰ Also, the molecular analysis found that *E. sativa* phytochemicals fused with multiple adhesion proteins associated with biofilm formation, causing compromised biofilm formation.¹⁹⁰ Advanced spectroscopic analysis showed that different bioactive compounds (eg, flavonoids, phenols, terpenes, and tannins) contribute to the versatile biological action of natural constituents, including the antibiofilm effect.^{189–192}

In conclusion, bacteria alter their gene expression by sensing the levels of molecules depending on the external environment, thus affecting biofilm production and other virulence factors.¹⁹³ Phytochemicals can regulate QS and, as a result, control biofilm-caused infections by controlling bacterial gene expression. The primary challenge of applying phytochemicals in antibiofilm therapy is the insufficiency of animal studies and clinical trials. Advancing research to these studies will accelerate their applicability in medicine.

Bacteriophages

Phages were introduced as a potential treatment for multi-drug resistant (MDR) pathogens, and several laboratories and research centers globally perform intensive bacteriophage research. Bacteriophages, in short phages, are bacterial viruses that can infect and kill or incapacitate specific kinds of bacteria but leave other bacteria and human cells unharmed.^{194–196} Phages are the most abundant biological entities in the biosphere, and they have been used therapeutically in some parts of the world since the 1920s.¹⁹⁷ Phages are under renewed consideration globally as tools for fighting the growing crisis of antibiotic-resistant bacteria.¹⁹⁸ The phage biology makes them potential candidates for phage therapy applications and other activities against multidrug-resistant (MDR) pathogens in general. Phage biology includes target selection where the phages, which have specificity in their cell infection, can infect the bacterial cell, propagate in the cell, and then lyse vast numbers of the cell.¹⁹⁹ Towards the end of the infection cycle, phage lytic enzymes destroy their bacterial host cell wall.²⁰⁰ As a phage-based therapeutic option, phage enzymes are exploited to eradicate bacterial virulence symptoms, such as biofilms.²⁰¹ Using these enzymes to treat bacterial infections associated with biofilms was suggested by destroying the biofilm matrix.²⁰² Phages can penetrate the biofilm layers through pores and channels and subsequently destruct the biofilm matrix. Phages encode various lysis enzymes, such as depolymerase, holins, and endolysins, which can degrade bacterial polysaccharides and rapidly destroy the integrity of biofilms to facilitate phage penetration of the cells within the inner biofilm layers.^{203,204} Depolymerases have been identified in 143 phages and classified in two classes: hydrolases and lyases, which recognize, bind, and digest EPSs to disturb biofilm structure.²⁰⁵ Endolysins are peptidoglycan hydrolases produced at the end of the infection cycle and cleave the peptidoglycans in the cell wall. Using phages and their derivatives against biofilms and MDR infections associated with biofilms align with developing safe and effective therapeutic strategies against biofilm-associated diseases.^{206–208}

Pei and Lamas-Samanamud engineered a T7 phage that overcame the limitation of affecting a host's biofilm and disrupt polymicrobial biofilms.²⁰⁹ This phage cleaves the biofilm signaling molecules in an approach known as quorum-quenching. Consequently, it aids in treating multiple hosts in a mixed biofilm infection.²⁰⁹ Silpe and Bassler reported that the *V. cholera* phage altered its response, lysis or lysogenic, based on the quorum-sensing molecules produced by the host (bacteria) and symbolized it as the phage having the power to “listen in” to the bacteria.²¹⁰ These QS signals are necessary for virulence and biofilm production. They suggested we could engineer phages to induce bacterial killing in the future, which can be advantageous in medicine, agriculture, and industry.²¹⁰

Several phage-based therapeutic options are available to prevent pathogenic bacterial biofilm formation. Figure 6 summarizes a few examples of potential applications of phages, eg, combining phages and antibiotics (eg, the sublethal concentration of ciprofloxacin and phage), engineering phages genetically (eg, knock-out virulence-encoding genes), using phage-derived enzymes (eg, endolysins), and utilizing phage cocktail therapy (mixture of phages).^{211,212}

Promoting phages in maintaining human health is imperative to developing novel, safe, and efficient treatments for antibiotic-resistant biofilm-mediated infections.²¹¹ The current limitations facing phage therapy include the shortage of local phage therapy Centers that specialize in isolating, identifying, and applying them to treat patients with acute MDR infections. In addition, resistance to phages is a rising issue that requires further exploration to provide better treatment.

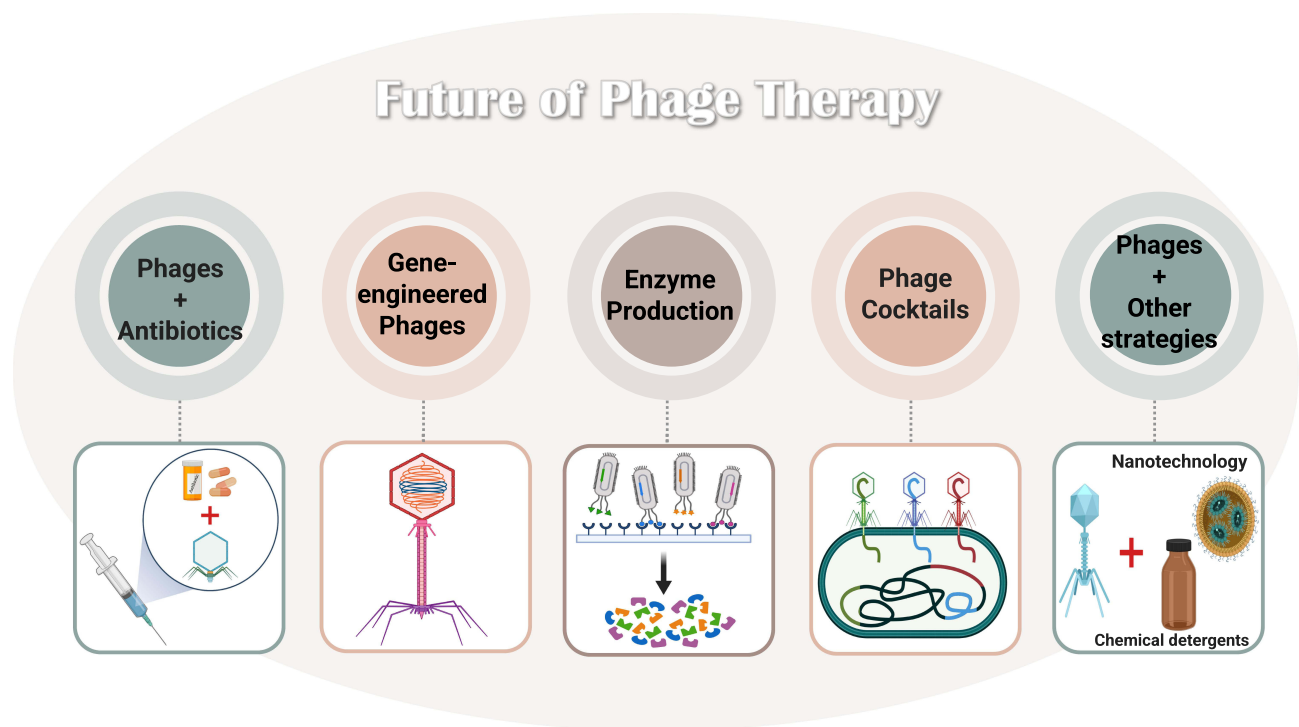


Figure 6 Future of phage therapy and the prospects of applying it to treat and prevent bacteria and their biofilm. Data from Liu et al.²¹¹

Bacteriophages and Nanotechnology

Combining phages and nanotechnology enhances phage infectivity, stability, and delivery. Esteban and others introduced Bacteriophage-K in an oil-in-water nano-emulsion formula to improve its stability and infectivity, which increased the bactericidal effect against *S. aureus*.²¹³ Interestingly, the phage-nano emulsion formula lowered the electrostatic repulsion between the phage and bacteria, both negatively charged, thus boosting the phage-bacterial interaction and improving antibacterial activity.²¹⁴ Magnetic phage-nanocomposite conjugates (PNCs) were used to disturb the biofilm's inner layers and to eradicate the bacterial biofilms. Small-sized PNCs removed both dual and multi-species bacterial biofilm with 98% and 92% elimination efficiency rates.²¹⁵ Yu et al estimated that this activity followed the equal dispersal of phages into the bottom layer of the biofilm.²¹⁵ In another study by Liu et al, phages were locally isolated, purified, and conjugated with chitosan film as a biocompatible agent to control bacterial infections and subsequent biofilm formation in medical implants.²¹⁶ The conjugated phage-chitosan film reduced bacterial growth and stimulated neural tissue regeneration in vitro.²¹⁶ Compared to the free phage, the antibacterial activity was less effective in the phage-chitosan conjugate, which is likely due to the controlled motion of the phage when conjugated. Still, it was estimated that the biofilm prevention resulted from lowering the bacterial density by the phage, 79.5% reduction compared to the control, based on SEM imaging.²¹⁶ To conclude, phages and nanotechnology can be applied as complementary strategic approaches to target bacterial biofilm formation.

Antimicrobial Photodynamic Therapy (aPDT)

Antimicrobial photodynamic therapy (aPDT) involves a combination of three key components. It requires a visible source of light to activate the photosensitizer appropriately with a specific wavelength of visible light, a non-toxic photosensitizer (PS), and the presence of ambient oxygen that is activated to produce the cytotoxic reactive oxygen species (ROS) and inactivation of the targeted cells (Figure 7). Recently, photodynamic therapy (PDT) studies emerged as a novel non-invasive therapeutic option, which is effective and most efficient for treating localized and superficial infections caused by bacteria present as biofilms, fungi, and viruses.^{217–219} Also, it is a unique procedure with interesting therapeutic approaches and applications in dentistry to treat biofilm-caused oral infections.^{217,220}

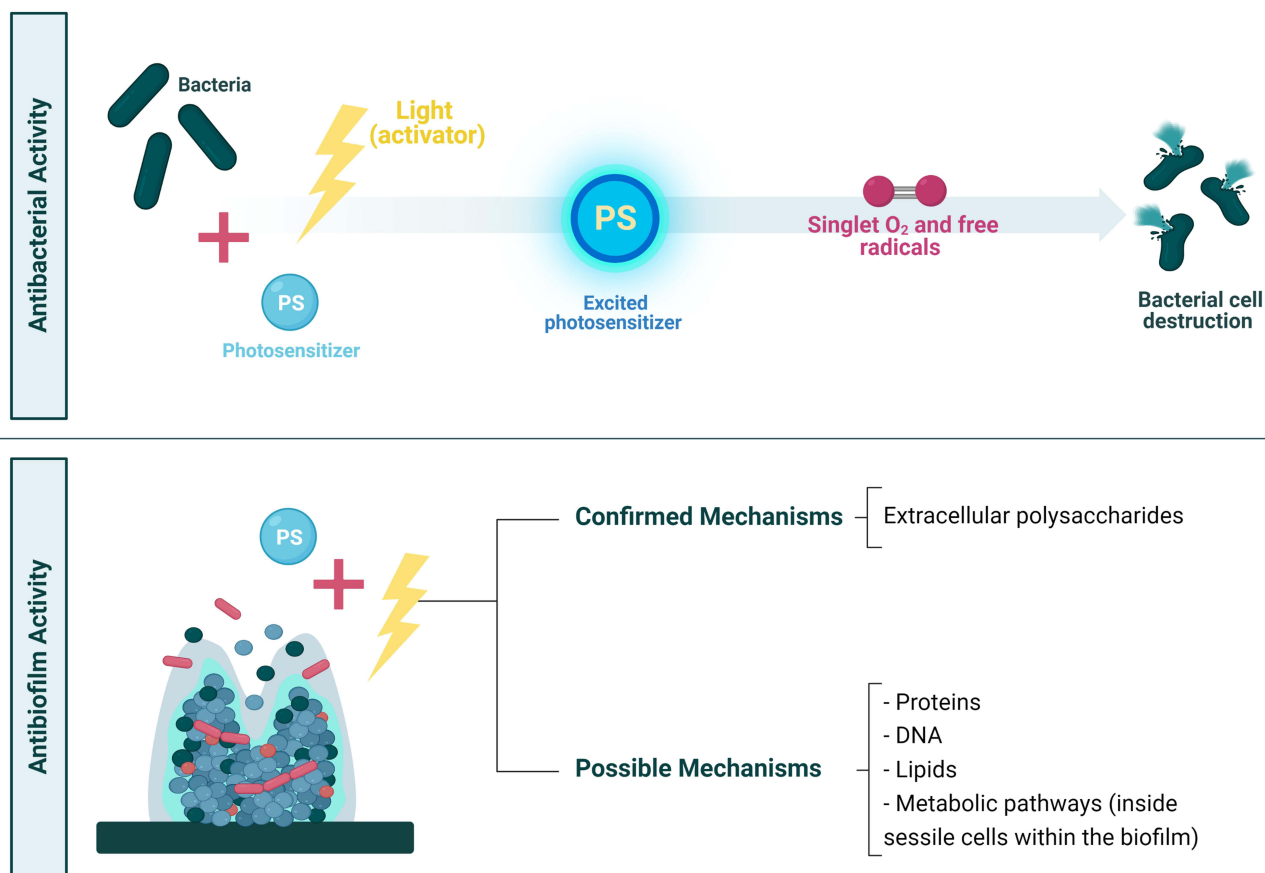


Figure 7 aPDT mechanism and application as an antibacterial and antibiofilm. The bacteria are absorbed into the photosensitizer and activated to an excited state post-exposure to light at a specific wavelength. Then, from light to molecular oxygen, the photosensitizer (PS) energy can be transferred. When bacteria are formed in a biofilm, aPDT could target the biofilm matrix comprising extracellular polysaccharides or other targets directed towards the bacterial cells within the biofilm.

Notes: Data from these studies.^{220,221}

Microorganism-Photosensitizer (PS) Interactions

The effectiveness of aPDT against different microorganisms depends on the photosensitizer (PS) type, concentration, and class of microorganism. The antibacterial property of aPDT is based on the idea that visible light induces the photosensitizer (PS), which produce ROS. The produced ROS destroys the bacteria unselectively via an oxidative burst, and the confirmed antibiofilm action impacts the integrity of the extracellular matrix (see Figure 7).^{220–222} The microorganism-photosensitizer interactions depend on the microorganism's physiochemical features, including relative solubility in water and lipids, constant ionization of factors for light absorption, and efficiently producing oxygen.²²⁰ aPDT is more effective against Gram-positive bacteria than Gram-negative bacteria, because of their porous cell wall composed of peptidoglycan and lipoteichoic acid. The PS can reach the cytoplasmic membrane and hinders the structural organization (forming its physical and functional barrier) in Gram-negative bacteria differently. Gram-negative bacteria possess a more complex morphology due to their negatively charged outer cell membrane, which comprises lipopolysaccharide, lipoproteins, proteins (with a porin function), and peptidoglycan. Notably, biofilm reduces the photodynamic activity of PS because of the structural difference in the cell membranes of the microorganisms within the biofilm. Another factor is the presence of other components, such as extracellular polysaccharide matrix and QS factors.^{218,220} Although numerous planktonic pathogens are killed by aPDT, the sensitivity of biofilm-derived anaerobic bacteria to aPDT is underexplored. The efficiency of aPDT requires microbial cell adsorption to the PS and penetrating the cell wall via subsequent activation through light irradiation.²²³ aPDT effectively reduces the viability of microbial cells and biofilms in cells and may be an important alternative therapy to traditional dental care techniques to treat many diseases. Photodynamic

antimicrobial therapy demonstrates relatively lower toxicity, can enable immediate dental care, has a low cost, multiple PSs are available for each form of the light source, and the treatment is risk-free for the patients.²²⁰

The key advantages and benefits of aPDT are their effects on bacteria with substantial differences in the mode of non-selective action, including multiple molecular targets such as proteins, lipids, and nucleic acids. There is a limited adverse effect profile and damage to host tissue. The aPDT exhibits tissue specificity; it allows for curbing many human infectious diseases with no drug resistance and few side effects following multiple therapy sessions.^{218,224}

Recent techniques tested the efficacy of combining antimicrobials and aPDT (Figure 8). There is no authenticated protocol for this synergy. However, Vanesa and others suggest that the preferred method is to incorporate aPDT with antimicrobial compounds (synergistically) or perform aPDT periodically associated with long-term antimicrobial drug treatment sessions.²²⁵ This synergism aims to enhance antibiotic activity by reducing the dose, toxicity, and treatment time. Also, the likelihood of bacterial resistance to aPDT-antibiotic treatment is low, and studies provided evidence of its ability to revert resistant strains to their susceptible state to specific antibiotics after exposure to sublethal doses of aPDT.^{225,226}

Gene Editing by CRISPR-Cas

Role of CRISPR-Cas in Controlling Bacteria

The Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) system is emerging as a safe, targeted, and practical approach to treating microbial infections and genetic diseases.²²⁷ It requires combining Nanomedicine with CRISPR and specific cleavage of the regulator of bacterial virulence (Cas 9) complex component sensing system, which evolved as bacterial defense mechanisms.^{227,228} The CRISPR-associated (Cas) genes/proteins expression in human-associated bacteria occurs in diverse Gram-positive and negative bacteria with variable distribution in the human body.²²⁸ The CRISPR-Cas System is present in bacteria and archaea, offering sequence-based adaptive immunity against phages, plasmids, and mobile genetic elements.²²⁹ CRISPR technique uses portions of the bacterial DNA. This method for gene editing was first introduced in 2012 in US and Swedish laboratories. As shown in Figure 9, this technique generates directed cuts in a genome when paired with a particular guide protein [ie, Cas9] by using fragments of bacterial DNA.²³⁰

The CRISPR-Cas structure comprises CRISPR RNAs (crRNAs) and the binding of crRNA, which is unique to the invasive pathogen sequence, resulting in the shredding of the target DNA/RNA sequence by Cas enzymes.²³¹ CRISPR-Cas systems function at a molecular level with adaptive mechanisms known as sequence-specific protection methods that detect invaders and dissect their genetic material.²³² This protection mechanism was systematized in three stages, starting with adaptation, as the long precursor of CRISPR locus (Pre-crRNA) is transcribed into crRNA, which is obtained from the invader's genetic material. The crRNA matures after being incorporated into a CRISPR array. Lastly, an "interference cascade complex" is activated to spot the invader sequence and cleave it by a specific RNA-guided endonuclease.^{233,234} Recent advances in experimental CRISPR-Cas system research focus on creating animal models and applying functional genomics to screen and develop innovative drugs targeting infectious, immune, and genetic diseases (Figure 10).²²⁷

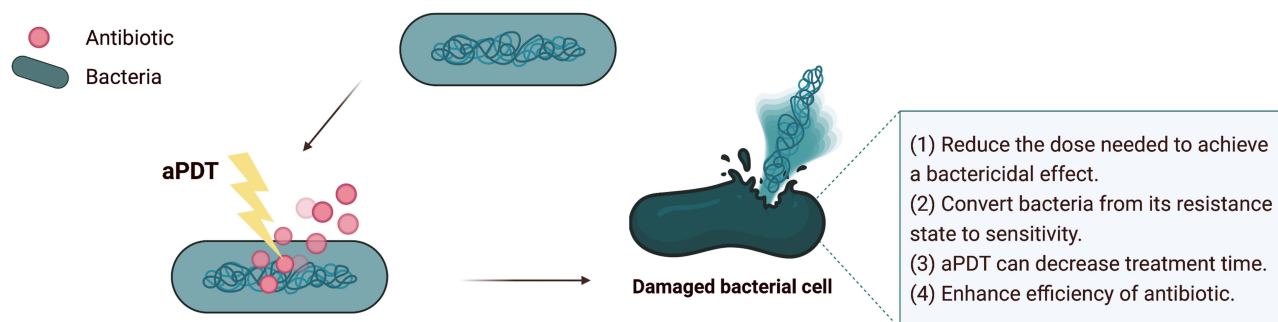


Figure 8 The synergism between aPDT and other antibiotics/antimicrobials.

Notes: Data from these studies.^{220,225}

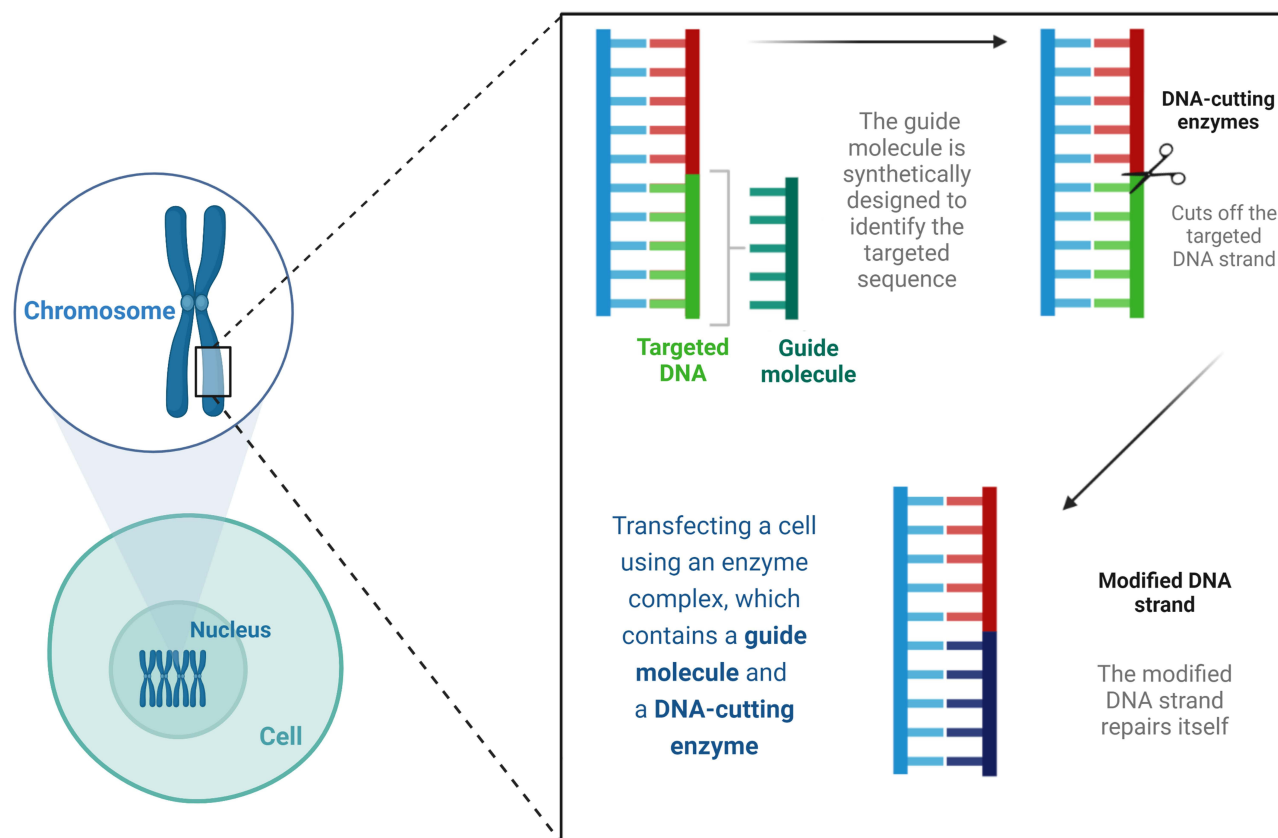


Figure 9 The CRISPR-Cas9 as a concept in gene editing.

Notes: Data from Ho C.²³⁰

Abbreviations: DNA, deoxyribonucleic acid.

Insights into CRISPR-Based Gene Editing to Control Biofilm Formation

The CRISPR-Cas system protects bacterial cells by identifying and cleaving any invading nucleic acids. It also interferes with bacterial species-specific competitions and the ability to produce several virulence factors during infection, such as regulating gene expression, developing biofilms, repairing the DNA, reacting to stress, and acquiring resistance genes. Scientists can alter the system to provide new insights to understand the role and application of gene editing and modulate CRISPR-Cas using anti-CRISPR agents or antimicrobials against various microorganisms.²²⁹

Zuberi et al introduced a novel concept of “CRISPR interference (CRISPRi)” and its role in inhibiting bacterial biofilm formation by targeting a gene essential for QS.²³⁵ CRISPRi inhibition produces distinct levels of targeted knockdown, which supports the study of behavioral changes in the bacteria when a gene is expressed at different levels. This study proposed that CRISPRi is a promising technique for inhibiting bacterial biofilm and controlling nosocomial and environmental infections by targeting the *luxS* gene and intervening with bacterial QS.²³⁵ In detail, the *luxS* gene is an essential gene that encodes for a vital QS molecule, the autoinducer-2 (AI-2) molecule, which has a role in biofilm formation and maturation.²³⁶

Research by Zuberi et al used CRISPRi to control urinary tract infections (UTI) and pathogens such as *E. coli*, specifically in regulating the adhesion property of the bacteria.²³⁷ Their idea was to target and knock down the *fimH* gene expression, which causes the suppression of transcriptional machinery through lodging inactive or “dead” Cas9 at a particular location. These results were assessed using a mannose-sensitive hemagglutination assay and TEM. The authors proposed that this approach be authenticated and applied in treating in vivo infection models to confirm its potential in UTI therapy.²³⁷

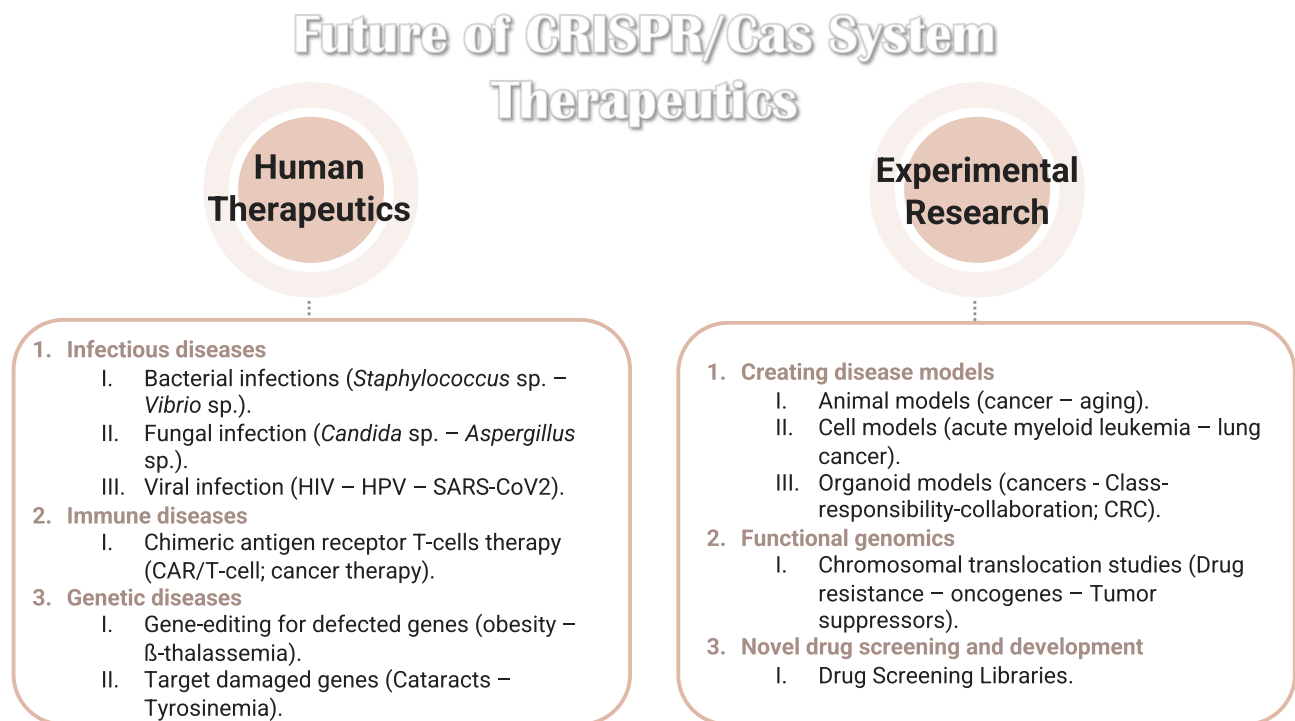


Figure 10 The recent experimental research on CRISPR/Cas system summarized the therapeutic of new approaches targeting bacterial virulence factors by applying nanomedicine to treat specific infectious and metabolic disorders.

Notes: Data from Dubey et al.²²⁷

Abbreviations: HIV, human immunodeficiency virus; HPV, human papillomavirus; SARS-CoV2, severe acute respiratory syndrome coronavirus 2; CAR, chimeric antigen receptor T cells; CRC, colorectal cells.

Noirot-Gros and others used CRISPRi to evaluate its influence on biofilm-associated genes in *Pseudomonas fluorescens*.²³⁸ Cyclic diguanylate (c-di-GMP), a signaling messenger responsible for many bacterial cellular functions, eg, virulence, was monitored with other regulators controlling biofilm formation. They found that CRISPRi inhibition of biofilm formation resulted from the deletion and silencing of genes expressing the biofilm's thickness and mass.²³⁸ CRISPRi also influenced gene expression at the operon level, such as the motility (swarming) and biofilm formation genes (eg, EPS-producing genes).²³⁸ A study applied CRISPR/Cas9 gene-editing system to *Cedecea neteri* (Gram-negative bacteria) isolated from the *Aedes* mosquitoes.²³⁹ The study aimed to mutate the outer membrane protein A (*ompA*) gene, resulting in an impaired ability to form biofilms and reduced infection prevalence compared to wild-type strains. Using this technique in the site-specific integration of genes will simplify the improvement of para-transgenic control methods to manage arthropod-transmitted infections.²³⁹

In short, the CRISPR system used in gene editing alters the gene expression level qualitatively or quantitatively.²⁴⁰ Controlling the gene expression of specific virulence factors, such as forming biofilm and secreting QS molecules, creates a new opportunity to treat different biofilm-associated diseases. Applying this approach to treat and diagnose bacterial infections requires more investigations regarding its pros and cons and how and when it can be applied to achieve its full potential.

Nano-Mediated Newly Advancing Approaches

Biofilms contain cells with phenotypic heterogeneity within a self-produced 3D matrix of EPS. The power of nanoparticles (NPs) to infiltrate the entire matrix enables them to engage with the cells within the EPS matrix, susceptible and resistant strains. Also, NPs interfere with the physicochemical interactions in the matrix, which are essential for keeping the stable 3D structure of biofilms.²⁴¹

Why are Nanoparticles Unique in Treating Bacterial Biofilm?

Primarily, the antibiofilm activity of nanoparticles results from their small size allowing their penetration into the biofilm microenvironment and effectively disrupting its integrity.²⁴² A study used silver-silica dioxide nanoparticles (AgSiO₂ NPs) as

an implant coating to inhibit *S. aureus* biofilm infections. Results showed size-dependent antibiofilm action; this effect was documented in the smaller NPs (6 nm) due to the amplified release of silver ions compared to the larger particles (11 nm).²⁴³ Research also investigated the antibiofilm activity of chitosan-capped gold and silver nanoparticles, biologically fabricated using the tiger milk mushroom (*Lignosus rhinocerotis*).²⁴⁴ The results showed more antibiofilm activity, 53.21% and 46.24% inhibition against *P. aeruginosa* and *S. aureus* biofilms, of smaller-sized metal nanoparticles (< 50 nm).²⁴⁴ Secondly, the larger surface area of nanoparticles enhances surface reactivity and increases the antimicrobial and antibiofilm action.²⁴⁵ Enhanced antibiofilm activity and a larger surface area have been verified in silica nanoparticles,²⁴⁶ metal and metal oxide nanoparticles, such as copper oxide nanoparticles,²⁴⁷ ferrite nanoparticles,²⁴⁸ silver nanoparticles,²⁴⁹ and titanium dioxide nanoparticles.²⁵⁰

Other factors related to the NPs also contribute to the antibiofilm action of NPs, such as hydrophobicity, shape, and surface charge.²⁵¹ Carefully considering these factors when designing antibiofilm NPs can aid the synthesis of NPs targeting bacterial biofilms. To destroy the bacteria inside the biofilm, NPs may inflict an antimicrobial action directly or deliver therapeutic agents, such as antibiotics or antimicrobials (eg, essential oils, enzymes, or phytochemicals).¹⁷

Metal nanoparticles can uniquely control the bacterial signaling system, which has been tested in vitro and in vivo.²⁵² Metal NPs block the synthesis of signaling molecules by interfering with the QS regulatory genes. They also can impede the access of signaling molecules into the neighboring cells by preventing them from binding to other cell's receptors or degrading these secreted molecules⁵ (Figure 11).²⁵² Another antibiofilm mechanism of silver NPs is integrating within the bacterial DNA and causing damage.²⁵³ In addition, gold NPs trigger ROS-mediated damage,²⁵⁴ while titanium oxide NPs (TiO-NPs) induce EPS

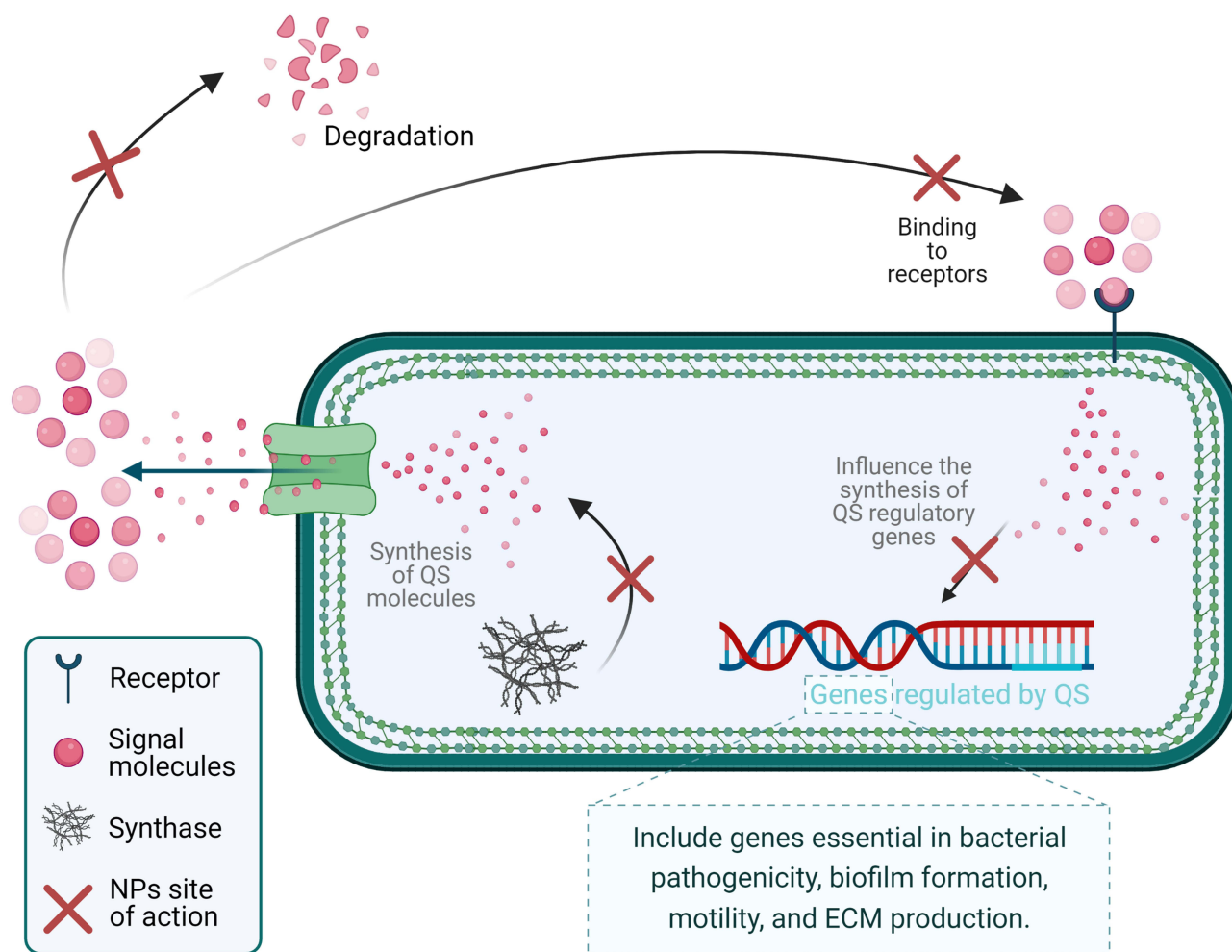


Figure 11 Schematic illustration of anti-QS mechanisms of nanoparticles.

Notes: Data from Qais et al.²⁵²

Abbreviations: QS, quorum-sensing systems; NPs, nanoparticles; ECM, extracellular matrix.

lipid-peroxidation.⁵ Antibiotic-loaded liposomes obstruct the bacterial electron transport system. Some NPs are synthesized to suppress QS, and chitosan NPs can invade the EPS matrix and destroy the biofilm due to their positively charged exterior.⁵

Recently, a new approach combined the CRISPR-Cas9 gene-editing technique and nanotechnology. Wan and others created a dissolvable patch to treat inflammatory skin disorders.²⁵⁵ They used nanoparticles to deliver the cas9 (gene editing agents) and glucocorticoids into cell nuclei to exploit their corresponding action. The in vivo results and mouse models showed reduced skin inflammation and enhanced glucocorticoid therapy.²⁵⁵ This study motivates research targeting pathogenic bacteria using gene-editing techniques to deliver NPs loaded with appropriate anti-virulence or repurposed antibiotics.

Nanoparticles: Classifications, Synthesis, and Applications

Antimicrobial NPs and nanomaterial coatings with good mechanical and tribological properties (ie, adhesion, friction, and wear resistance) are novel approaches to treating biofilm infections.^{5,243} NPs are divided in four groups according to their chemical composition: carbon, inorganic, organic, or hybrid NPs.²⁵⁶ This review will focus on the inorganic NPs, metal and metal oxide NPs, and organic NPs (liposomes).

Inorganic nanoparticles (metal and metal oxide NPs) have an inherent antimicrobial activity with a multimode action. They also have an increased antimicrobial effect compared to their bulk form, which allows them to be used either as inhibitors or as carriers for antimicrobials with synergistic effects.^{257,258}

Jang and others synthesized bimetallic (silver and copper) NPs integrated on graphene oxide nanosheets to study their antibiofilm activity.²⁵⁹ They tested the safety and efficacy of this nanocomplex in vitro and in vivo. Biofilm formation was reduced in *P. aeruginosa* and in vivo assays proved their safety on human dermal fibroblasts. Lastly, an animal model with induced wound infection recovered within seven days when the nanocomposites were applied topically.²⁵⁹

Recently, scientists used inorganic nanoparticles using various capping agents. Khalid and others used functionalized silver and iron oxide NPs (35 and 48 nm) to treat biofilm development and formation of *S. aureus* and *P. aeruginosa*.²⁶⁰ Rhannolipids were used as ligands to increase NPs-bacterial interactions and enhance antimicrobial activity. The results showed high efficiency in inhibiting bacterial adhesion and biofilm formation (>80%). Khalid et al hypothesized that this activity ensued from the synergetic action of ROS production by the metal NPs and the reduced bacterial-adhesion affinity caused by rhannolipids.²⁶⁰ El-Batal and others chemically synthesized magnesium ferrite nanocomposites loaded with amoxicillin and stabilized them with citric acid to test their antimicrobial and antibiofilm ability.²⁶¹ The nanocomposites inhibited > 90% of *S. aureus* and *E. coli* biofilm. A membrane leakage assay and scanning-electron microscope displayed a perforated bacterial membrane post-treatment. The researchers suggest using this nanosystem as a surface disinfectant as they excelled in the UV-light excitation tests.²⁶¹ However, Padmavathi et al found that capping copper oxide nanoparticles decreased their antibiofilm activity; the non-coated particles completely eradicated the biofilm of copper-tolerant *Staphylococcus lentus*, unlike the cetyl trimethyl ammonium bromide-capped NPs.²⁶² They proposed that the capping of the particles restricted the release of the copper ions, thus reducing its efficiency.²⁶²

Researchers assessed using biosynthesized or green metal nanoparticles in several applications as biosensors, antimicrobial, antioxidant, and anticancer therapy.²⁶³ When metal particles reduce to the nanoscale, it results in intrinsic and unique physiochemical (eg, size, shape), biological, and optical properties.²⁶³ The diverse targeting system of NPs lowers the likelihood of bacteria gaining resistance in the future.^{264,265} Though the green synthesis of NPs is an eco-friendly, non-toxic, and cost-effective alternative to conventional methods, its application has limitations, including stability, toxicity, purity, and the absence of universal synthesis/waste regulations.^{256,266}

In conclusion, inorganic NPs represent a novel approach to treating infections caused by biofilm-forming bacteria. Exploring the aspects of functionalizing the surfaces of these particles or utilizing green practices will boost NP stability, purity, and activity. Risk management of NPs toxicity and setting environmental regulations to handle and dispose of nanomaterials correctly are required to avoid the negative consequences of a thriving technology.

Organic NPs are nanosystems harboring carbon macromolecules (eg, liposomes) used in drug design and delivery, image-channeled treatments, and imaging.^{267,268} Liposomes are vesicles with a membrane comprising a bilayer of lipids with an aqueous core, which present an innovative technology to carry effective molecules to the targeted sites. The “active” loaded aqueous substance can be hydrophobic or hydrophilic.^{269,270} Though these particles excel due to their low toxicity and high drug entrapment ability, their use is challenged by their low target specificity, instability, and short half-life in serum.^{267,271} To

overcome these disadvantages, scientists functionalized these particles using ligands (eg, aptamers (Aptamosomes)^{272,273} or polyethylene glycols (PEGylation)^{271,274}). In detail, aptamosomes are liposomes conjugated with aptamers—a short portion of RNA or DNA designed to improve specificity and affinity.²⁷³ These conjugates have been studied recently for cancer therapy or imaging.^{275,276} More research is vital to explore the ability to formulate unique antimicrobial aptamosomes.

Utilizing polyethylene glycols (PEGylation) in the liposomal formulation was done to improve the efficacy and delivery and decrease the toxicity of the beta-lactam antibiotic nafcillin.²⁷¹ PEGylated nafcillin liposomes (PEG-Lipo-NF) were compared to non-functionalized nafcillin liposomes (Lipo-NF), and the free nafcillin against Methicillin-susceptible *S. aureus* (MSSA). The PEG-Lipo-NF had higher antimicrobial activity than the Lipo-NF or the free antibiotic. The PEG-Lipo-NF had the lowest MIC ($0.25 \pm 0.01 \mu\text{g/mL}$) and improved biofilm inhibition activity (MBIC₅₀ $0.5 \pm 0.02 \mu\text{g/mL}$). Also, in vivo and animal studies showed that PEGylated nafcillin liposomes reduced NF-toxicity and improved cellular viability and liposomal availability.²⁷¹ In another study, Das and scientists evaluated the dual activity of a PEGylated formula loaded with doxorubicin, epigallocatechin gallate (EGCG), and quercetin against *E. coli* and a human lymphoblastoid cell line (K-562) as an approach to control chemotherapy-associated secondary microbial infections.²⁷⁴ The PEGylated formula had a loading efficacy of $70.8\% \pm 5.25$ and released the drugs gradually for ten days. The combination of the antioxidant (EGCG) and doxorubicin increased liposomal stability and led to cancer cell death by apoptosis, necrosis, and ROS formation. The antibacterial effect might have resulted from the encapsulated EGCG and quercetin, as the latter have a cell wall damaging ability.²⁷⁴

Liposomes have been studied as vehicles for QS inhibitors, the communication signals between bacteria in biofilms. Liposomes loaded with QS inhibitors showed a dose-dependent activity, higher than unloaded liposomes. This activity persisted and lasted up to 48 hours, unlike the free biofilm inhibitors that lost their effect.²⁷⁷ Studies showed that polyethylene glycol-modified and cationic liposomes could be highly effective antibiofilm agents against *P. aeruginosa*. The findings demonstrate that the efficacy is linked to the modification level and the liposome's surface charge.²⁷⁸

Nanotechnology is a promising method. Recent studies suggest combining it with other anti-virulence and anti-QS drugs to achieve ultimate activity while decreasing the probability of future resistance.^{279–281} Organic and inorganic NPs exhibit superior physical, chemical, mechanical, thermal, and biological properties applied in medicine for their antimicrobial, anti-inflammatory,

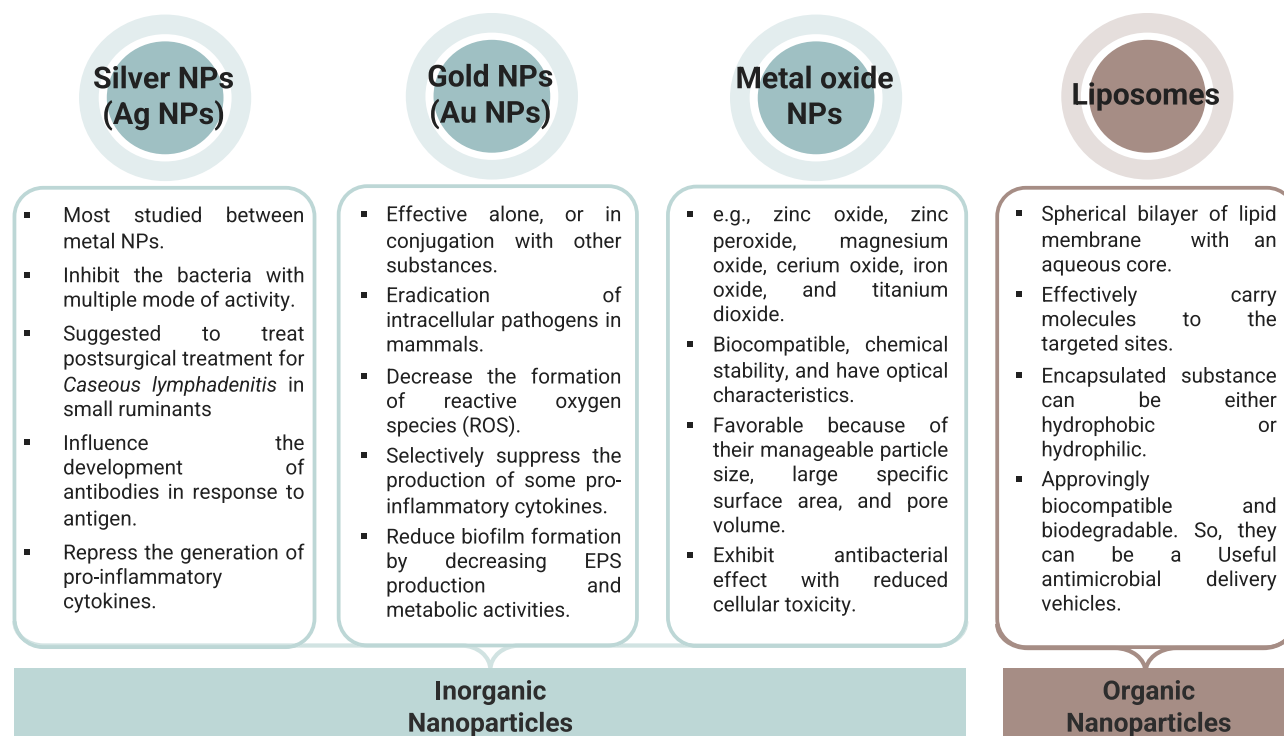


Figure 12 Types of nanoparticles and their application.

Abbreviations: NPs, nanoparticles; Ag NPs, silver nanoparticles; Au NPs, gold nanoparticles; EPS, extracellular polymeric substance.

and antioxidant mechanisms. These NPs are extensively used due to their high stability, multi-functionality, biocompatibility, adhesive and therapeutic properties, and broad applications, as shown in Figure 12.^{2,5,241,257,264,282,283}

Conclusion

This review discussed biofilm formation in pathogenic bacteria, its association with virulence factors (such as QS systems), antibiotic resistance, and future therapeutics. *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* are prevalent biofilm-forming bacteria causing a variety of community and hospital-acquired infections. Targeting bacterial virulence factors, eg, biofilm formation, is a promising approach that aims to destroy the bacterial cell without inducing selective pressure, which is the source of the global resistance issue. The only limitation of such an approach is its dependency on host immunity to fully eradicate the infection. This limitation is especially an issue for immunocompromised individuals. Another possible solution suggests co-administering anti-virulence drugs (eg, antibiofilm) with low doses of antibiotics to attenuate the bacteria by disrupting their biofilm and amplifying the antibiotic's activity. Therefore, reducing the excessive use of antibiotics, resetting bacterial resistance, and restoring its susceptibility. Although this approach is promising, the results take time. We explored other approaches in this review: inventing novel drugs by repurposing old drugs. Natural compounds, phages, and the CRISPR/Cas technique have proven their role in regulating QS systems and interfering with biofilm formation by regulating gene expression. aPDT is a novel method that damages the integrity of bacterial cell walls and the biofilm matrix. Recent advances in nanomaterials use NPs with multimode activity or combined with other therapeutics, such as phages, phytochemicals, and antibiotics, to manage biofilm-caused infections. Researching innovative methods should achieve maximal efficacy and specificity with minimal toxicity, ensure long-term therapeutic effects, and lower the production cost of valuable drug formulations to control infectious diseases. In addition, advancing these studies to in vivo experimentation and to clinical trials is recommended to prove the capability of these approaches in treating biofilm-associated infections.

Abbreviation

agr, accessory gene regulator genes; AHL, N-acyl homoserine lactones; AI, autoinducers; aPDT, antimicrobial photodynamic therapy; Cas, complex sensing systems; CRISPR, Clustered Regulatory Interspaced Short Palindromic Repeats; ECM, extracellular matrix; eDNA, extracellular DNA; EPS, extracellular polymeric substances; HAI, hospital-acquired infection; MBIC, minimum biofilm inhibitory concentration; MDR, multi-drug resistance; NP/NPs, nanoparticle; PS, photosensitizer; QQ systems, quorum quenching systems; QS systems, quorum sensing systems.

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Disclosure

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