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Review Article

Novel therapeutics and diagnostics in mono- and polymicrobial biofilm infections: Addressing emerging challenges

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Abstract

Microbial biofilm infections are a major challenge in modern medicine. They can be monomicrobial, consisting of a single species, or polymicrobial biofilms, in which the bacterial and fungal species coexist. They form highly structured and resilient communities that can coordinate through quorum sensing, modulate metabolism, and produce protective extracellular matrices. These properties allow biofilms to persist on tissues and abiotic surfaces and can cause serious infection involving chronic wounds, osteomyelitis, endocarditis, cystic fibrosis lung infections, diabetic foot ulcers, catheter-associated urinary tract infections, prosthetic joint infections, and ventilator-associated pneumonia. Their capacity to resist antibiotic exposure and evade host immunity can cause prolonged disease, recurrent infections, and is also one of the main contributors to the global healthcare burden. This review aims to explore novel therapeutic and diagnostic strategies, focusing on the critical gaps limiting current clinical management and traditional treatment outcomes. For that, through recent literature, the current diagnostic and therapeutic limitations and emerging solutions were analyzed. The findings highlight that the efficacy of traditional antibiotic activity in planktonic and biofilm is completely different. Biofilms exhibit significantly enhanced resistance and tolerance mechanisms. For that, new and more effective alternatives are urgently needed. Advanced strategies include combination therapies, nanotechnology-based antimicrobials, engineered phages, anti-quorum-sensing agents, antimicrobial peptides, and CRISPR-Cas approaches. Parallel progress in diagnostics, including molecular techniques, advanced imaging, biosensors, and biomarker discovery, offers improved detection of biofilm-associated pathogens. These innovative strategies towards the early detection and advanced therapeutics show strong potential to transform the management of biofilm-associated infections.

Keywords: Biofilms, Antimicrobial Resistance, Antimicrobial Therapy, Diagnostic Tools, Clinical Management, Therapeutics, Microbial Communities.

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Introduction

Naturally, microbes live in biofilms. A biofilm is a community of microorganisms embedded within a self-produced matrix, attached to a living or non-living surface.

Free-floating bacteria attach to a surface and begin to multiply. As they proliferate, they secrete a protective matrix composed of hydrated sugars, proteins, and DNA, known as an extracellular polymeric substance (EPS). EPS acts as a protective barrier, physically shielding micro-organisms from environmental stress such as antiseptics, antibiotics, and the host's immune system. Biofilm can spread across the surface by releasing individual biofilm cells or by releasing new EPS protected colonies. That's why they are difficult to remove completely, even following aggressive debridement, and can reform [1]. Within 24 to 48 hours, a mature biofilm can form. They can quickly evolve, adapting to conditions and becoming more robust and mature [2].

On a structural basis, biofilms can be categorized as monomicrobial or polymicrobial. Monomicrobial biofilms consist of a single microbial species. They are typically simpler in structure, which makes them easier to study and target therapeutically. For example, *Pseudomonas aeruginosa* forms single-species biofilm during infections in individuals with cystic fibrosis [3]. Polymicrobial biofilms include more than one species, i.e., a combination of bacteria and fungi. They can communicate through quorum sensing, forming a very resilient and adaptive network [4]. Polymicrobial biofilms cause most of the chronic infections. To understand the severity of infections and develop effective treatments and accurate diagnoses, we must first examine their structure and composition in depth.

Biofilm-related infections are emerging as major global health care system clinical and economic challenges. They are involved in chronic infection, including chronic wounds, cystic fibrosis, lung infections, and urinary tract infections. These infections are commonly linked to medical devices such as catheters, prosthetic implants, and ventilators. This results in high morbidity and mortality, especially in immune-compromised patients. They are responsible for approximately 80% of the chorionic infection. It is also proven that bacteria in a biofilm can be 10 to 1,000 times more resistant than their free-floating bacteria [5]. Their protective layer of extracellular polymeric substance enables them to survive antibiotic treatments and to become antimicrobial-resistant (AMR). This physical barrier also helps them evade the host's immune system by blocking the immune cells from crossing the barrier and targeting the microbes. These factors highlight the urgent need for improved diagnostic tools and innovative therapeutic strategies [6].

Beyond the health impact, it also imposes significant economic burdens on healthcare systems. Prolonged hospital stays, repeated interventions, and extended antimicrobial therapy can increase overall treatment costs and loss of productivity. It is estimated that biofilm-related infections contribute to a significant proportion of hospital-acquired infections worldwide [7]. The global cost estimates for biofilm-related diseases and medical devices are shown in Table 1 from previously published economic analyses.

This review aims to provide an overview of emerging therapeutic and diagnostic approaches for biofilm infections. By analyzing recent studies on innovative drug delivery systems, combination therapies, biofilm-targeting molecules, and advanced diagnostic tools, this review summarizes existing knowledge to inspire the development of novel solutions to overcome the current challenges posed by biofilm infections.

Pathophysiology and clinical challenges of mono- and polymicrobial biofilm infections

A biofilm develops through a series of steps that shape its clinical behavior.

Table 1: The global cost estimates for biofilm-related diseases and medical devices (PHS consulting Ltd, 2021) [7].

Sector (Medical & Human Health)	Global ($bn)	Comment
Wound healing	281	Biofilms form on wound surfaces and delay healing.
Cystic fibrosis	7.5	Lung mucus in cystic fibrosis patients is colonized by pathogens forming biofilms.
Infective endocarditis	16	Biofilms on natural and artificial heart valves cause serious cardiac disease.
Chronic sinusitis	24.4	Often associated with secondary biofilm infections that are difficult to clear.
Ophthalmology	0.759	Eye surfaces are prone to biofilm formation.
Human antibiotics	34.2	Many bacterial infections linked to biofilms are treated with antibiotics.
Central venous catheter bloodstream infection	11.5	Biofilms colonize catheters and lead to bloodstream infections.
Catheter-associated urinary tract infection	1	Biofilms colonize urinary catheters, causing infections.
Prosthetic cardiac valves and pacemakers	0.22	Surgically implanted devices may host biofilms, often requiring surgery.
Ventilator-associated pneumonia	2.3	Biofilms on endotracheal tubes can cause pneumonia and prolonged hospital stays.
Breast implants	0.093	Implant surfaces can become infected with biofilms.
Prosthetic joints	7.8	Implanted joint surfaces may host biofilms, often treatable only by surgery.
Total medical and human health	386.8	—

The process begins with the attachment of the microbial cell to a surface that can be a cell, tissue, or medical device. After attachment, they begin to multiply and form micro colonies that consist of a few cells. The microbes present in the colony secrete an extracellular matrix composed of polysaccharides, proteins, lipids, and extracellular DNA, which acts like a protective layer. It provides biofilm structure and creates a nutrient channel that helps the community grow and mature. Once the biofilm becomes mature, the cells start to disperse to a new location and start the cycle again, as illustrated in Figure 1 [1, 8].

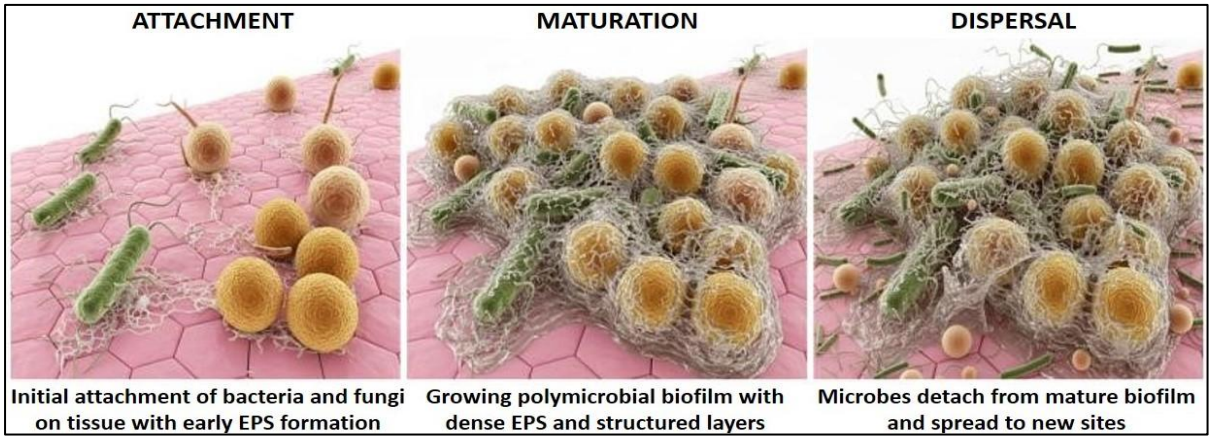

Figure 1: The biofilm life cycle showing the main stages involved in biofilm development.

The pathophysiology of biofilms is directly linked to whether it is monomicrobial or polymicrobial. Monomicrobial biofilms coordinate through species-specific quorum sensing. For example, *Pseudomonas aeruginosa* uses Las, Rhl, and PQS systems to regulate the formation and dispersal of the biofilm matrix. *Staphylococcus aureus* utilizes the agr system to regulate virulence factors and matrix production [9]. Polymicrobial biofilms have far greater complexity as they consist of different microbial species, including bacteria, fungi, or sometimes algae. They involve cross-species quorum sensing, metabolic cooperation, co-aggregation, and division of labor. They can exchange signaling molecules, like autoinducer-2 (AI-2), farnesol, or short-chain fatty acids that help them to communicate. This communication network develops coordination for biofilm thickness, matrix composition, stress responses, and also results in synergistic pathogenicity. For example, *Candida albicans* secretes farnesol to enhance the antibiotic tolerance of *Staphylococcus aureus*. Farnesol increases the expression of efflux pump genes, i.e., *norA, norB, and norC*, and makes them vancomycin resistant [10]. Similarly, anaerobes in chronic wound biofilms reduce oxygen levels, creating microenvironments that favor the survival of other pathogens [11].

Once a biofilm is established, it exhibits multiple mechanisms to evade the host immune system. The most important one is the secretion of extracellular polymeric substance (EPS) by microbial cells. It is composed of polysaccharides, proteins, lipids, and extracellular DNA (eDNA). EPS acts as both a physical and chemical barrier that prevents immune cell penetration and antibiotic diffusion. It creates a microenvironment in which biofilm cells are shielded from external stress. Multiple layers of cells are formed within this matrix due to the metabolic heterogeneity, gradients in oxygen, nutrients, and waste products. Cells in the periphery layer remain metabolically active, while in deeper layers, cells adopt a dormant state. These dormant cells, often referred to as persister cells, express stress-response proteins (catalases, superoxide dismutases) that protect them from reactive oxygen species produced by phagocytes. Reduced metabolic activity also lowers antigen presentation, which results in decreased recognition by toll-like receptors (TLRs) and other immune sensors [12]. Biofilms further evade immunity through active secretion of immunomodulatory molecules, like *Staphylococcus aureus*, which produces staphylococcal superantigen-like proteins (SSLs) and chemotaxis inhibitory proteins (CHIPS) to block neutrophil chemotaxis and complement activation [13]. *Pseudomonas aeruginosa* secretes rhamnolipids and pyocyanin that can induce neutrophil apoptosis and impair oxidative burst. These contribute to the chronicity and recurrence of biofilm infections [14].

One of the most significant clinical challenges of biofilm-associated infections is antimicrobial resistance (AMR). Biofilms exhibit phenotypic tolerance as the matrix restricts drug penetration or neutralizes reactive oxygen species; the cells survive antimicrobial exposure [15]. Enzymes such as β-lactamases and aminoglycoside-modifying enzymes are trapped within the EPS, and they degrade antibiotics before they reach deeper layers [16]. Biofilms also promote horizontal gene transfer through close cell proximity and abundant eDNA, accelerating the acquisition of plasmid-borne AMR genes [17]. Prolonged infection and continued exposure to the same antimicrobial can also lead to genetic adaptations, like mutations in efflux pumps, stress response regulators, and quorum-sensing networks that further enhance resilience. Biofilms on surfaces like catheters, implants, and ventilators can remain asymptomatic for weeks before causing severe systemic infections. Chronic

wounds, diabetic ulcers, cystic fibrosis lungs, and osteomyelitis are often caused by the polymicrobial biofilms that respond poorly to conventional antibiotics. This makes biofilm infections one of the most challenging clinical problems in modern microbiology and medicine.

Advances in therapeutics

Conventional approaches and limitations

Antibiotics remain the first-line therapy for suspected bacterial infections, but when microbes organize into biofilms, especially polymicrobial biofilms (bacteria + fungi, or multiple bacterial species), these therapies often fail. Several factors contribute:

(i) The extracellular matrix produced by biofilm acts as a physical and chemical barrier, limiting diffusion of the antimicrobial agent. Antibiotics must diffuse through this dense, sticky mesh of polysaccharides, proteins, and extracellular DNA; their concentration decreases as they move deeper into the layers. As a result, cells in the interior are exposed to sub-therapeutic drug levels.

(ii) Chemical gradients of oxygen, nutrients, and pH create zones of metabolic heterogeneity that reduce drug efficacy. Some species may produce enzymes (e.g., β-lactamases), and inter-species interactions can enhance matrix robustness, increase EPS production, resulting in resistance.

(iii) Gradients of oxygen, nutrients, and pH create zones where some cells are actively growing, while others, often deeply embedded, shift to a dormant "persister" state. And many conventional antibiotics target active processes, so persister cells survive.

(iv) Polymicrobial biofilms, the presence of diverse species, bacteria, and fungi together, complicates therapy. Drugs effective against bacteria may not affect fungi, and vice versa [18].

Clinically, this can result in treatment failure and often persists despite prolonged or high-dose therapy. These limitations are summarized conceptually in Table 2, which compares the typical efficacy of antibiotics against planktonic versus biofilm-associated microbes.

Table 2: Comparative efficacy of conventional antibiotics against planktonic bacteria and their effects on biofilm.

Antibiotic class	Planktonic efficacy	Biofilm-associated efficacy	Typical fold-reduction in activity	Ref
β-lactams	Good (if susceptible)	Poor / often ineffective	~ 10-1000× less effective	[19]
Aminoglycosides	Usually bactericidal (Gram-negatives)	Much reduced / tolerance common	~ 640-fold increased resistance	[20]
Fluoroquinolones	Strong (DNA-target, broad)	Variable, often reduced killing, needs a higher dose	~ 10-500× lower efficacy in many cases	[21]
Glycopeptides (e.g., vancomycin)	Effective vs planktonic Gram-positives	Often poor in biofilms (especially device/tissue-associated)	Significantly reduced efficacy	[22]
Polymyxins / membrane-disrupting agents	Active vs planktonic Gram-negatives	Some activity, but high doses/toxicity needed	Reduced due to EPS/tolerance mechanisms	[23]

Emerging antimicrobial therapies

Because of the failure of conventional therapeutics, researchers are increasingly exploring novel strategies for biofilm treatments. The following subsections review the major promising approaches.

Combination therapies

One strategy is combining conventional antibiotics with agents, like compounds with therapeutic properties that disrupt the biofilm matrix or degrade polysaccharides or extracellular DNA, agents that alter matrix integrity, or may "loosen up" the biofilm, restoring antibiotic access. In Polymicrobial biofilms, this can be especially useful because matrix disruption affects the community structure overall, not just one species. When applied together, such combination therapies exhibit synergistic effects, resulting in a decrease in biofilm biomass, a dramatic decrease in viability, and even a reduction in persister populations. This dual approach is attractive because it can use existing antibiotics (well-known safety profiles) while overcoming biofilm-specific barriers. Like plant-derived bioactive metabolites show strong antibiofilm activity and can synergize with conventional antimicrobials to overcome resistance in *S. aureus*, *P. aeruginosa*, and *C. albicans* biofilms, offering promising alternatives for persistent infections [24 - 25].

Combining different antibiotics can also evaluate their efficacy against the biofilm. In a study, they combined multiple commonly used antibiotics in pairs, including ciprofloxacin-daptomycin, ciprofloxacin-vancomycin, daptomycin-tobramycin, and tobramycin-vancomycin against the clinical and standard isolated strains of *Staphylococcal aureus.* All combinations inhibited and disrupted biofilms in a concentration-dependent manner, with most achieving complete biofilm inhibition at 100× MIC and 77-97% disruption of preformed biofilms. Time-kill assays at 100× MIC reduced stationary-phase CFUs by 2-6 log10, though persisters remained. Overall, antibiotic combinations significantly reduced persister cell fractions compared to monotherapies ($p < 0.05$), highlighting their potential for clinical application against persistent staphylococcal infections [26].

Nanotechnology-based solutions

Nanoparticles (NPs) and nanocarriers offer a powerful tool to deliver antimicrobials deep into biofilms. Due to their small size and tunable surface chemistry, NPs can diffuse more effectively through the EPS matrix. They can carry antibiotics, antimicrobial peptides, enzymes, or even gene-editing tools, and release them locally in a controlled fashion, also sometimes triggered by environmental cues (pH change, enzymes, heat, and light).

For example, silver nanoparticles (AgNPs), known for their broad antimicrobial activity, have been incorporated into wound dressings or coatings for implants, showing potent disruption of bacterial biofilms (including multidrug-resistant strains) [27]. Liposomal antibiotic formulations encapsulate drugs inside lipid bilayers, improving drug stability, prolonging release, and helping drugs penetrate the biofilm more deeply than free drugs. Such nanocarriers can target bacterial and fungal cells simultaneously and can be especially valuable in polymicrobial biofilms [28].

Phage therapy

Bacteriophages (viruses that infect bacteria) are being repurposed as antibiofilm agents. Phage therapy can target multiple bacterial species in polymicrobial biofilms. Upon infection, phages replicate and lyse their host cells. Many phages also produce depolymerases or endolysins that degrade components of the EPS matrix, weakening the biofilm structure and

facilitating further phage or antibiotic penetration. Advances in genetic engineering enable the creation of genetically modified phages with enhanced host range, modified depolymerases, or even payloads (e.g., antimicrobial peptides or gene-editing tools). This adaptability makes phage therapy particularly promising for complex, multi-species biofilms where standard antibiotics fail [29].

Anti-quorum sensing (QSI) agents

Biofilm formation and maintenance often rely on cell-to-cell communication (quorum sensing, QS). QS regulates EPS production, virulence factor secretion, and maintenance of biofilm architecture. Disrupting QS with antiquorum-sensing agents (QSIs) can block these signals, reducing EPS production, impairing biofilm stability, and sometimes triggering dispersal. As a result, bacteria become more susceptible to antibiotics or immune clearance. They can be small molecules, enzyme inhibitors, or analogues. QSIs target communication rather than vital cellular processes; they may exert lower selective pressure for resistance. Like Benzaldehydes inhibit *P. aeruginosa las* and *pqs* systems by over 80% and also improve the antibiofilm activity of ciprofloxacin and tobramycin [30].

Antimicrobial peptides (AMPs)

AMPs are short, often cationic peptides that disrupt microbial membranes, interfere with biofilm structure, and sometimes modulate host immune responses. Their small size and amphipathic nature enable them to penetrate EPS effectively. In many studies, synthetic or engineered AMPs reduced biofilm biomass, killed persister cells, and worked against diverse species, bacteria and fungi alike, making them attractive for polymicrobial infections. In vivo studies (e.g., wound infection models, pulmonary infection models) show that AMPs can reduce microbial burden and inflammation, illustrating translational potential. Recently, macrocyclic peptide antibiotics like zosurabalpin have shown potent activity against carbapenem-resistant *A. baumannii* by inhibiting the $LptB_2FGC$ complex and disrupting lipopolysaccharide transport, offering one of the first truly effective therapeutic options for this highly drug-resistant, biofilm-forming pathogen [31].

CRISPR-Cas based antimicrobials

A cutting-edge approach involves using gene-editing systems (e.g., CRISPR-Cas) to selectively target microbial genes critical for biofilm formation, antimicrobial resistance elements, or virulence factors. Delivered via nanoparticles, phagemids, or conjugative plasmids, CRISPR systems can disrupt biofilm-forming capacity or re-sensitize bacteria to antibiotics. In mixed-species or polymicrobial biofilms, the specificity of CRISPR allows targeting particular species or resistance determinants, minimizing off-target effects on other beneficial microbes. Though still largely experimental, this strategy offers a highly precise and customizable antibiofilm tool [32].

Physical and mechanical approaches

In addition to chemical or biological strategies, physical and mechanical methods are also being developed to disrupt biofilms or prevent their formation, particularly useful for medical devices and implants.

Ultrasound and electromagnetic-based disruption

Low-frequency ultrasound (sonotherapy) can disrupt the biofilm matrix mechanically. Microstreaming, cavitation, and shear forces loosen the EPS, create micro-channels, and enhance antibiotic penetration. When combined with

antibiotic therapy or drug-loaded nanocarriers. Ultrasound significantly increases drug delivery into biofilms and enhances bacterial kill rates. This approach has shown promise in an animal model of device-associated infections and wound biofilms. In an in-vitro study, curcumin-mediated SPDI achieves up to approximately 6.9-log reduction in biofilm viability [33].

Some studies also explore electromagnetic fields (EMF) or pulsed electromagnetic fields (PEMF), which may perturb microbial membrane potential or ionic balance, destabilizing biofilm integrity or enhancing susceptibility to antimicrobials. Though still early, such methods hold promise, especially for surface-associated biofilms on implants [34].

Surface modifications

Perhaps the most practical long-term approach is prevention, modifying the body's surfaces to resist biofilm formation. Passive antifouling coatings (hydrophilic polymers, zwitterionic brushes, PEG-based coatings) reduce initial microbial adhesion. Active coatings, such as those releasing antimicrobial agents (silver, antimicrobial polymers), can kill or inhibit microbes on contact. For polymicrobial biofilms, coatings that combine anti-adhesive and antimicrobial actions are especially beneficial, as they prevent simultaneous colonization by bacteria and fungi [35].

Summary of emerging antimicrobial strategies targeting biofilm-associated infections are mentioned in Table 3.

Table 3: Summary of emerging antimicrobial therapies for the control of biofilm-associated infections.

Category	**Emerging Therapy**	**Key Scientific Details (Mechanism & Advantage)**
Enhanced Drug Action	Combination Therapies	Uses antibiotics synergistically with agents (e.g., enzymes, chelators).
	Antimicrobial Peptides (AMPs)	Cationic molecules that interact electrostatically with microbial cell membranes, causing direct lysis
Targeted Delivery & Disruption	Nanotechnology-Based Solutions	Utilizes nanoparticles and nano-carriers (like liposomes or silver nanoparticles) for targeted, site-specific delivery of antimicrobials.
	Anti-Quorum Sensing (AQS) Agents	Compounds that interrupt the Quorum Sensing (QS) bacterial communication system are necessary for biofilm maturation, architectural maintenance, and virulence factor production
Biological Control	Phage Therapy	Uses natural or genetically engineered bacteriophages that specifically target and lyse the bacterial components of the biofilm
	CRISPR-Cas Systems	Gene-editing technology delivered to microbes to specifically target and disrupt genes vital for biofilm formation or antibiotic resistance
Physical & Mechanical Aids	Ultrasound and Electromagnetic Fields	Applies physical energy to create mechanical stress on the biofilm structure, leading to matrix disruption.
		Can enhance the overall penetration of co-administered antibiotics.
	Surface Modifications	Engineering medical device materials (e.g., catheters) with anti-adhesive properties or sustained-release antimicrobial coatings

Advances in diagnostics

Limitations of conventional diagnostics

Diagnostics for common infections rely on culture-based methods, as they are effective for many acute infections for detection. But they have serious drawbacks when it comes to biofilm-associated infections. Biofilms often comprise polymicrobial communities, multiple bacterial species, sometimes bacteria and fungi combined, embedded in a protective extracellular matrix. So, the standard culture does not detect diversity within the biofilm because many microbes within biofilms are slow growing, metabolically dormant, or in non-culturable states. As a result, culture often fails to detect all participating microbes and can give false results. Clinical studies of chronic wounds and device-associated infections report that culture-based methods miss significant fractions of pathogens that are now revealed by molecular methods. Also, culture ignores spatial structure, metabolic heterogeneity, and biofilm-specific phenotypes. It cannot tell whether detected microbes are part of a structured biofilm or if they are expressing biofilm-associated genes or any specific resistance gene. So, this limitation can lead to underdiagnosis, misdiagnosis, or inappropriate therapy [36].

Novel diagnostic tools

Imaging techniques

The Advancement in imaging technologies enables the direct visualization of biofilms. It allows detailed visualization of biofilms in vitro, ex vivo, or even in tissue samples. Through imaging, one can observe biofilm structure, cell layers, matrix density, and cell viability gradients. Confocal laser scanning microscopy (CLSM) allows researchers to observe biofilms in real time. Confocal laser scanning microscopy (CLSM) combined and multiplex FISH to visualize polymicrobial biofilms in chronic wounds [37]. Results revealed complex 3D structures and multiple microbial phenotypes that were undetectable by standard methods.

Molecular diagnostics

Molecular diagnostic techniques such as PCR, metagenomics, and next-generation sequencing (NGS) are a few examples. They have broadened the way to detect and characterize microbial communities. PCR provides rapid and species-specific detection even when microbial load is low. qPCR adds the ability to quantify target organisms and can also measure the expression of biofilm-related genes. Metagenomics and NGS extend this further by profiling the entire microbial population, including all different microbial organisms that traditional culture often misses. These sequencing approaches can identify antimicrobial resistance genes, virulence factors, and even strain-level variation that gives a more complete picture of the infection. Findings of a study on chronic wound and implant-associated infections increasingly show that molecular testing reveals complex, polymicrobial communities and resistance determinants that would have gone unnoticed under culture-based testing. Another advantage is that molecular diagnostics can detect DNA from dormant or non-culturable cells, sometimes providing an early warning before clinical symptoms become obvious [38].

Biosensors

Biosensor technologies enable the real-time detection of early biofilm formation. They use the biological elements that can produce a certain signal to detect. For example, electrochemical sensors can detect microbial adhesion, metabolic activity, or redox changes before a mature biofilm develops and allow pre-emptive intervention [39]. Electrochemical biosensor array functionalized with DNA

probes targeting 16S rRNA of common uropathogens have been developed. They analyzed urine samples from 116 patients within 1-2 hours of collection, and the results were compared with standard clinical culture methods. The biosensor achieved pathogen detection sensitivity of 89%, specificity of 100%, and identified twenty microbes, including *E. coli*, *Pseudomonas aeruginosa*, and *Enterococcus* species. This demonstrated the feasibility of rapid, multiplexed pathogen identification using biosensors to improve early diagnosis and clinical management of biofilm-associated infections.

Biomarker discovery

Biomarker discovery means finding unique molecules to biofilm growth. They offer a sensitive alternative. Researchers have identified many candidate biomarkers such as EPS components (matrix proteins, extracellular DNA-binding proteins), Stress-response proteins expressed by dormant cells, biofilm-specific metabolites, and quorum-sensing molecules. These molecules can be detected in body fluids, such as wound exudate, blood, and catheter effluent, and allow biofilm diagnostics.

Multimodal studies revolutionize the discovery of biomarkers [40]. The infection-specific patterns of gene expression can be identified by transcriptomic studies, such as ESTs, SAGE, DNA microarrays, and RNA-Seq. It enables identification even when pathogens are embedded within biofilms. On the other hand, proteomic studies identify unique proteins expressed during infection by using techniques like DIGE (Differential In-Gel Electrophoresis) and iTRAQ (isobaric Tags for Relative and Absolute Quantitation). Metabolomic profiling further enhances detection by analyzing metabolites produced through cellular metabolism (Figure 2). That can be detected using techniques such as CE and TOF-MS to capture infection-specific metabolites. All of these approaches help in

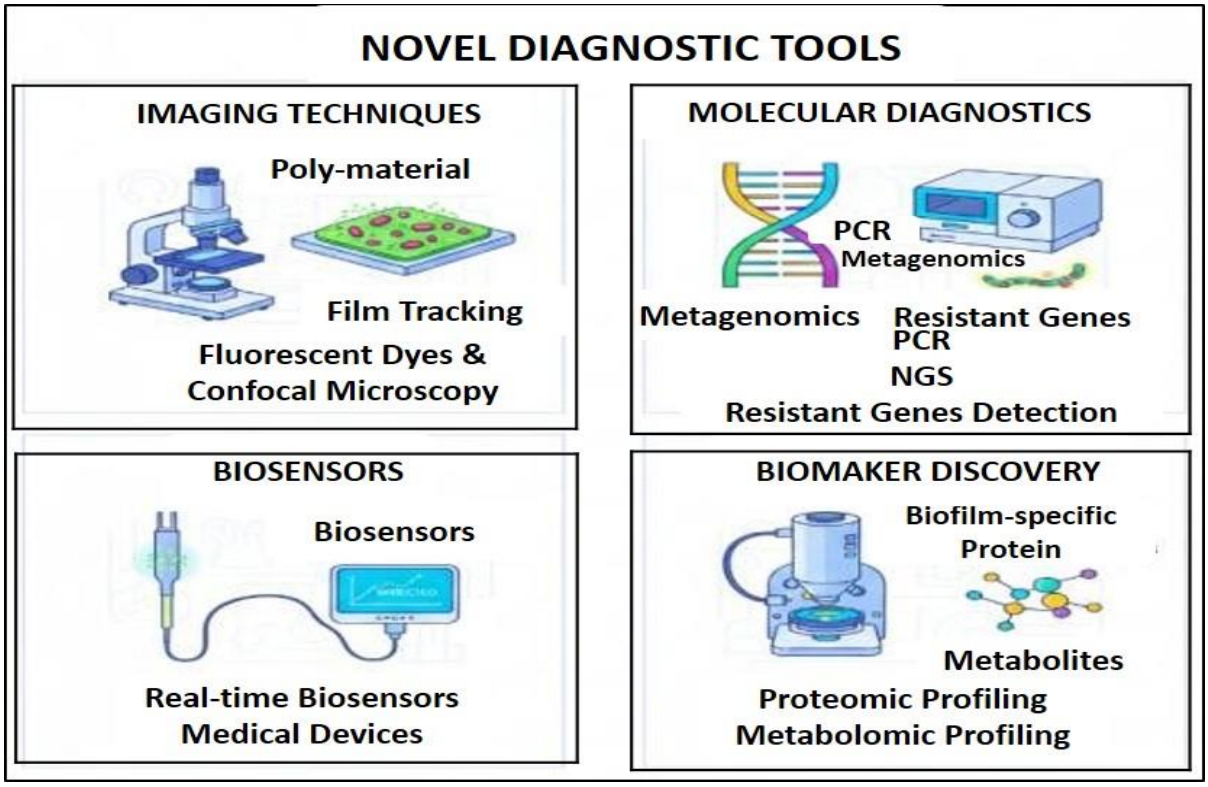

Figure 2: Some novel diagnostic approaches used for the detection and characterization of biofilms

Rapid, sensitive and species-specific detection of pathogens involved in biofilm-associated infections [41]. Recently, the *tesG* gene expression was discovered as a predictive biomarker for chronic *P. aeruginosa* pulmonary infections by examining the 210 sputum samples from patients with chronic *P. aeruginosa* lung infections [42].

Integrated therapeutic and diagnostic platforms (Theragnostic)

Recent advancements in technology have enabled the development of theragnostic platforms. It is an integrated system that can diagnose the presence of biofilm as well as deliver targeted therapy. Such as biofilm-targeting nanoparticles conjugated with both antimicrobial and diagnostic labels e.g., fluorescent dyes, MRI contrast agents, or radionuclides. After administration, these nanoparticles accumulate at biofilm sites due to the ligand-matrix interactions. The fluorescent label allows real-time imaging of biofilm load, while the therapeutic cargo releases antimicrobial agents in situ to eradicate the biofilm. This dual action enables monitoring treatment efficacy and also adjusting therapy accordingly. Several in vitro and in vivo studies have been conducted on developing such systems. Scientists used a biofilm-responsive nanoparticle (MBP-Ce6 NSs) that releases imaging and therapeutic agents inside MRSA biofilms. It provided both fluorescence detection and also reduced biofilm bacteria by ~2.5-log with no toxicity [43]. In another study, nanoparticles functionalized with biofilm-binding peptides were loaded with fluorescent markers for detection and antibiotics for killing microbes that successfully reduced *P. gingivalis* biofilm biomass [44].

Another advancement in diagnostics is point-of-care (POC) diagnostic devices. These are portable diagnostic instruments that can be used to detect the patient rapidly without visiting laboratories. It reduces the delay between the severity of infection and treatment initiation. It includes microfluidic chips, biosensors, and portable imaging probes that can facilitate early diagnosis. For example, POC microfluidics for *E. coli* O157:H7 was developed using methods like SPR, RCA, and electrochemical sensing. These systems provide early-stage screening and make them valuable for routine testing [45]. Following the progress made in point-of-care tools, Lab-on-a-Chip (LOC) devices have grown, bringing key lab functions onto a single chip. These can also help to detect biofilm pathogens by combining micro-scale environments with imaging, optical, or electrical sensing to measure bacterial accumulation and activity in real time. A BiofilmChip device was developed for testing biofilm formation and the effect of antibiotics on it. It was also tested for *P. aeruginosa* and *S. aureus* grown using a sample directly taken from a cystic fibrosis patient. Biofilms were formed and visualized using confocal microscopy [46].

Challenges and future directions

The strong protection and invasive nature of microbes in a biofilm make it really challenging to treat and diagnose. They can attach to any moist or dry surface, including living or non-living ones. They are enclosed in an extracellular matrix and are responsible for many chronic infections. Biofilm infections are difficult to treat and detect due to the diverse community. They often respond poorly to standard therapies and require a deeper understanding of their dynamic development and complex nature.

One of the core therapeutic difficulties in biofilm-associated infection is the development of unique antimicrobial resistance mechanisms. The antimicrobial resistance mechanism in biofilms is different from traditional genetic resistance. Studies have shown that antimicrobial resistance can evolve faster in

biofilms compared to in planktonic cells [47]. Very high levels of ciprofloxacin were subjected to *E. coli* biofilms, and they rapidly developed resistance within a week. The EPS matrix shields microbial cells from antibiotics, host immunity, and environmental stress. It can trap positively charged molecules, such as aminoglycosides, or can degrade them through secreted enzymes. Host eDNA and neutrophils are also trapped in it, which further reinforces this shield, in chronic infections like those caused by *Pseudomonas aeruginosa*. High cell density and limited antibiotic penetration create conditions that reduce the efficacy of the drug. The effect is further amplified by persister cells that survive antibiotic exposure. Close cell-to-cell contact promotes the horizontal transfer of antimicrobial resistance genes. The matrix not only protects cells but also maintains the biofilm structure and supports nutrients that enhance survival. These interactions cause biofilms to evolve very quickly and lead to the spread of antimicrobial resistance [15].

Diagnosing biofilm infections remains challenging because clinical tests detect the presence of planktonic cells, rather than the community of microorganisms. Culture methods are unable to detect infection severity and most organisms in biofilms do not grow in normal laboratory conditions. Advanced techniques like confocal microscopy or mass spectrophotometry can be used to visualize and identify biofilms. But these techniques cannot be used regularly in clinical practice as these methods are expensive, labor-intensive, and impractical. Molecular techniques like PCR and sequencing provide a better detection of microbial diversity. But it can still not easily distinguish between active and inactive cells or provide information about the biofilm's physical structure. The biggest gap lies in the limited ability of current diagnostics to accurately detect polymicrobial interactions within the biofilm. Without identifying how species cooperate within a biofilm, we cannot predict how the community will respond to therapy [48].

Biofilm behaves differently between patients depending on the infected site, species composition, host immunity, and history of antimicrobial exposure. A single therapeutic approach cannot be universally successful. Precision strategies, such as species-specific drug combinations, quorum-sensing inhibitors, enzymes that degrade the matrix, or targeted drug delivery systems, are increasingly recognized as essential. But their clinical use remains limited due to cost, regulatory hurdles, and the lack of comprehensive diagnostic tools to guide such decisions. For example, susceptibility-guided therapy has been shown to improve *H. pylori* eradication that was of high clarithromycin resistance [49]. In another study of *Pseudomonas aeruginosa* from cystic fibrosis patients, biofilm-grown isolates showed unique antibiotic-specific resistance patterns independent of planktonic susceptibility. It highlights the potential value of biofilm-based susceptibility testing for personalized therapy [50].

In the drug development process, for testing any drug therapy, in vivo models matter. Real tissues have blood, an immune response, mucus, and nutrient gradients, and all of this can change how biofilms form and respond to treatment. They actually show how a drug behaves within a living system, how pathogens are affected by it, and the body's response. These are parameters that couldn't be studied in in vitro models. Currently, many in vivo models have been developed for tissue-related infections to study the complexity of the biofilms within the body. But these models are costly, ethically limited, and poorly replicate human immunity [51].

Advancements in new technologies can

help to overcome the current challenges associated with biofilm-related infections. AI is also emerging as a powerful tool for optimizing therapeutic strategies. Algorithms are made that can predict thousands of antimicrobial combinations, identify synergistic drug pairs, and design peptides to penetrate the extracellular matrix. In diagnostics, deep learning models have shown promise in automating the interpretation. By using biofilm microscopy images as training data, it can predict accurate results that reduce the time and expertise required for accurate identification [52]. Deep learning-based AI was applied to detect *Pseudomonas aeruginosa* biofilms with high accuracy using bright-field microscopy images. A pipeline developed using deep learning was used for biofilm detection as a pixel-wise segmentation problem. The dataset of 184 microscopic images of *Pseudomonas aeruginosa* (both planktonic and biofilm states) is fed to a U-Net architecture to exploit deep residual learning and improve the feature. Images with manually annotated biofilm were divided into ~150 training and 34 test samples. Results showed that the model achieved 86.62 % accuracy and 71.12 % precision [53]. In this way, AI may also help to improve biofilm detection, guide targeted treatment strategies, or predict how specific biofilms will respond to different interventions [54].

Such advanced technologies may help in the development of more effective, sustainable, and accessible solutions for the current challenges. As many advanced antibiofilm technologies, including engineered enzymes, nanomaterials, and complex imaging tools are expensive, resource-intensive, and impractical for low-income settings. This limits their real-world impact, especially where chronic wound infections and device-associated biofilms are most prevalent. Low-energy physical treatments, including ultrasound- or light-assisted approaches, offer additional alternatives that are safer and easier to scale. Ensuring that future biofilm interventions are affordable, easy to store and transport, and compatible with minimal laboratory infrastructure is essential for improving global health outcomes. There is growing interest in eco-friendly and cost-effective solutions such as natural antibiofilm compounds, phage therapy, and biodegradable coatings for medical devices [55]. A recent study developed an affordable, eco-friendly electrochemical microfluidic device for real-time biofilm monitoring. The device was built using 3D-printed molds followed by polydimethylsiloxane casting. It had electrodes that enabled impedance-based measurement of biofilm growth. It was tested on *Staphylococcus aureus* and *Candida albicans*. Biofilm formation showed clear sigmoidal growth patterns that were confirmed by SEM imaging. With high sensitivity (10.83 µA/CFU/mL) and a low detection limit (0.208 CFU/mL). Approaches like these may offer a practical and non-invasive alternative for studying biofilms in healthcare and environmental settings [56].

Conclusion

Biofilm-related infections are a clinical challenge. They can be established in living tissues or on any medical devices. These factors contribute to high morbidity and mortality among patients and increase the healthcare burden. They have several characteristics, such as quorum sensing (QS)-mediated coordination, interdependence of the metabolism, and an extracellular polymeric material matrix that limits the diffusion of antibiotics and makes the biofilm resistant. As the biofilms contain a mixed species population, there is no universally accepted detection system for biofilms. Most of the laboratories still rely on traditional microbiology designed for planktonic cells. This shows the urgent need for better clinical management to adopt novel strategies. Major progress is being made in diagnostics, like molecular

or high-resolution imaging in and targeted therapeutic agents, including antibiofilm molecules, bacteriophage therapy, for biofilm-associated infection. Furthermore, the integration of the detection and therapeutic delivery system develops theragnostic platforms that are capable of both diagnosing and delivering targeted therapy. For sustained future progress, developing a more precise medicine framework, engineering drug-eluting biomaterials, and involving predictive computational models is needed to discover or design high-throughput discoveries. Interdisciplinary collaboration is essential for developing more effective and clinically viable strategies against both mono- and polymicrobial biofilm infections.

Conflicts of interest

The authors reported no conflicts of interest.

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