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Microbial Biofilms: Where Are We and Where Are We Going?

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ABBREVIATIONS IBC, intracellular bacterial community; IMD, implantable medical device; LE, lipid emulsion; QS, quorum sensing; ROS, reactive oxygen species; YFP, Yellow Fluorescent Protein

KEYWORDS bacteria; biofilm; definition and challenges; fungi

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Historical Perspective

The discovery of biofilms is attributed to the father of microbiology, Antonie van Leeuwenhoek (1632–1723), when he was examining his own dental plaque and noticed the presence of "animalcules."[2] However, it was not until the 1970s that it became widely accepted that bacteria in all-natural ecosystems lived in the biofilm state. This timeframe coincided with research focusing on the biodeterioration of "Dhows," a lateen-rigged ship with 1 or 2 masts, used in the Indian Ocean in the 70s, in the Arabian Gulf, where upon examination of the hull of these ancient boats, researchers focused on the slime layer or microfouling, which turned out to be biofilms.[3] The association of biofilms with indwelling medical devices (IMDs) as they relate to infections was recognized in the early 90s when microscopic examinations revealed the presence of many microbes, mainly bacteria, enveloped by extracellular matrix. This realization, however, was not acknowledged as an important cause of IMD infections until the early 1990s when electron microscopic examination of explanted IMDs, believed to be the foci of infection, revealed large numbers of bacteria encased in a thick extracellular matrix.[4] This discovery led to a rapid increase in the number of researchers investigating biofilm-related IMD infection.

Recent studies brought to the forefront that gut resident bacteria and fungi residing in the gastrointestinal tract interact to form biofilms.[5] Biofilms formed by beneficial microbes are helpful to our gut lining. In contrast, biofilms formed by microbial pathogens are detrimental and could potentially exacerbate inflammatory symptoms, becoming resistant to antimicrobial drugs and immune cells. Similar interkingdom interactions have been observed in sites other than the gastrointestinal tract. In this regard, studies investigating chronic wounds observed that mixed-species bacterial (e.g., *Citrobacter freundii*) and fungal (*Candida albicans*) biofilms form rapidly with *Candida* forming the biofilm core, while bacteria are associated with the biofilm boundary. These findings propelled researchers to investigate approaches to manage biofilms.

What Are Biofilms?

The formation of microbial communities on natural surfaces, in chronic wound infections, in medical device buildup, and in dental plaque all share a common denominator: biofilms. Biofilms are an aggregation of bacteria and/or fungi surrounded by a self-produced extracellular matrix. This matrix gives rise to the main impediment in treating biofilms, because it makes the microorganisms inside highly resistant to antimicrobials and host defense mechanisms. The unique features and appearance of the biofilm are highlighted in Figure 1.

Biofilms in intravascular catheter-associated infections as well as diseases such as periodontitis, cystic fibrosis, and otitis media[5] are linked to several pathogenic fungi; however, the most prevalent fungi found within these conditions are the *Candida* species.[6]

There are several reasons why *Candida*, and especially *C albicans*, are the main contributors to fungal biofilms. Their ability to adhere to various surfaces as well as each other is facilitated by a unique class of proteins called adhesins, which have repeatedly shown significantly higher adhesion and cohesion abilities.[7,8] They also possess the trait of dimorphism, which is a key component in biofilm production because it allows them to effectively maneuver the change between yeast and hyphal growth.[9] Within a biofilm, *C albicans* also has an intense resistance mechanism, making it extremely resistant to antifungal treatments, and finally it has a complex system for metabolic adaptation that allows it to thrive in diverse environments.[10]

When looking at IMDs in particular, several characteristics define a traditional biofilm-induced IMD infection. These characteristics have multiple similarities with what has been observed in biofilm-related infections, including delayed onset of symptoms, inability of the

Figure 1. Visualization of a biofilm, using scanning electron microscopy. (A) The image displays the dense layers of co-aggregating yeast as well as hyphal forms. (B) Fungi embedded in the extracellular polymeric material; the image highlights the amorphous granular appearance of the extracellular material.

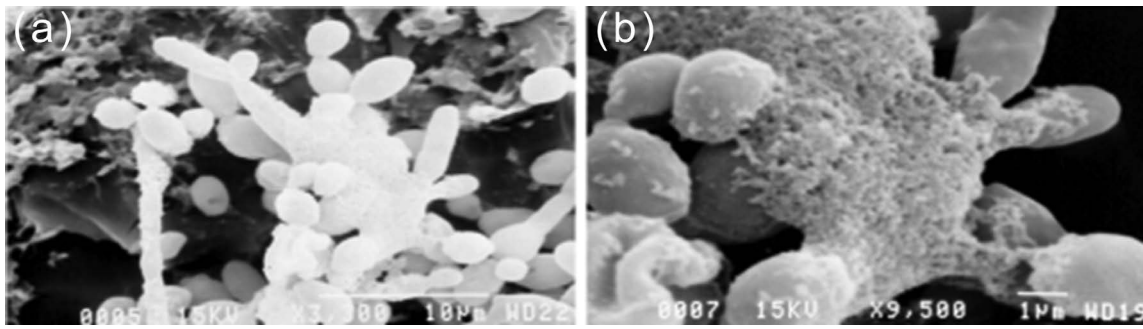


Table 1. Summary of Biofilm Components

No.	Components	Percentage (%)
1	Microbial Cells	2–5
2	Water	Up to 97
3	Polysaccharides	1–2
4	Proteins	<1–2 (including enzymes)
5	DNA and RNA	<1–2

host defense mechanisms to inhibit them, programmed detachment acting as a nidus for infection,[11] and inability of antimicrobials to significantly affect the biofilm. Biofilms are a proven major contributor to IMD-related infections, with the main route of treatment being the removal of the device. This method, however, is risky for the patient and is often not recommended because IMD removal requires surgery, which can damage the tissue surrounding the device. In addition, there is also a psychological component to be considered, as a surgery to remove a device used to control a critical and chronic condition usually has drawbacks. Moreover, IMD removal is also expensive, costing an average of 5 to 7 times more than IMD insertion.

The good news is that current research on biofilms has come a long way. With an increase in IMD insertions and removals has come a greater need for understanding the underlying mechanisms behind biofilms, how biofilms can be diagnosed, how they can cause other diseases, and how they are prevented and treated. While a vast amount of research has been conducted in several of these areas, there is still a great need for more dialogue on the topic, as well as more research on the treatment methods for patients in general.

An Overview of Biofilm Composition

Biofilms are microbially diverse structures composed of a mixture of bacteria and/or fungi. Current literature suggests that up to 80% of bacteria and archaeal life can be found within biofilms.[12] Biofilms develop an extracellular matrix of polysaccharides, protein, and extracellular DNA to protect the microorganisms within the matrix from a host of problems. This matrix gives the microorganism the ability to survive at lower oxygen and nutrient availability, osmotic shock, and gives a layer of protection against antimicrobials.[13] The top layer contains the bulk of oxygen and nutrients, which decreases gradually toward the center of the biofilm, sometimes allowing for certain anaerobic bacteria to survive at the center.[13] A summary of the composition of biofilms is listed in Table 1.

Most of the biofilms contents are suspended in water, while components of the extracellular matrix average about 1% to 2% each.[14]

The composition and formation of biofilms can also be better understood when looking at the complete biofilm life cycle, as described in Figures 2 and 3.

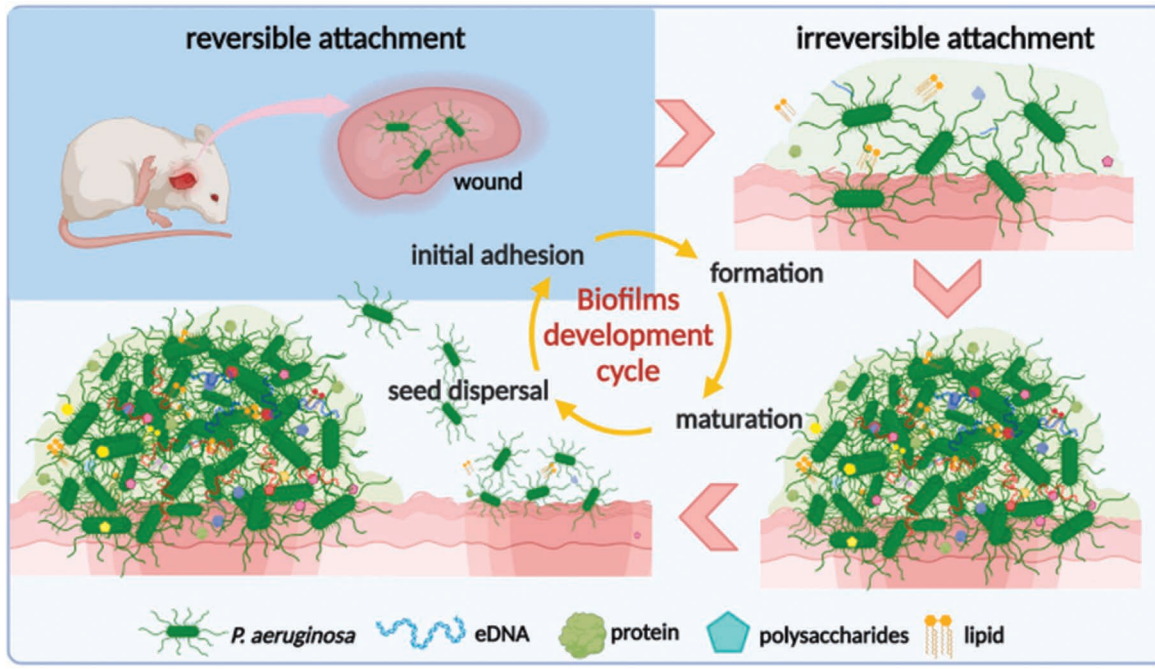
To test whether biofilms can act as a nidus of infection, biofilms were formed on catheters, using YFP (Yellow Fluorescent Protein)-tagged *Candida*. After 3 days, kidneys were aseptically harvested and examined microscopically. Immunofluorescence microscopy showed that YFP-tagged *Candida* was colonizing the kidneys in a fashion similar to the catheter. This suggests that eliminating the biofilm is critical to treat the catheter as well as biofilms formed internally (i.e., systemically).

The 3-dimensional structure of biofilms can vary depending on the bacterial species involved. For example, *Streptococcus pneumoniae* biofilms form in a linear pattern, while *Pseudomonas aeruginosa* biofilms adopt a mushroom shape.[13] In fact, the environment is a significant contributor to the overall biofilm 3-dimensional shape, because the local conditions allow the biofilm to adapt.[17]

Biofilms in Human Disease

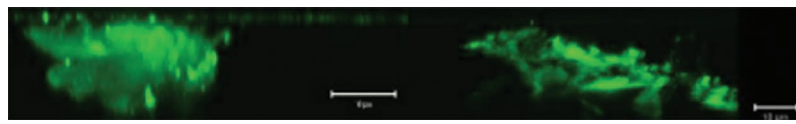
Around 75% of the infectious diseases found in humans can be attributed to biofilms.[18] Owing to the structure of biofilms, especially the matrix, the microorganisms within are much more resistant to

Figure 2. Stages of biofilm formation. Biofilm formation proceeds in 4 different steps: 1) Reversible attachment is where the microbes can attach onto a surface and is in a dynamic state where it is possible for it to return to its plankton form. 2) Irreversible attachment is when the microbial community gains more structure, and the matrix that allows the microorganism to thrive is formed. 3) Maturation phase is where the biofilm develops its 3-dimensional form to best fit the environment. 4) Microbial cell dispersal occurs when the biofilm has accumulated enough volume to cause nutrient deficiencies in the inner layer, which eventually results in a central cavity, allowing microbial cells to disperse.[15]



eDNA, Extracellular DNA.

Figure 3A. The displayed imaging shows YFP-tagged *Candida* as present on the catheter (A) and the kidney.



YFP, Yellow Fluorescent Protein.

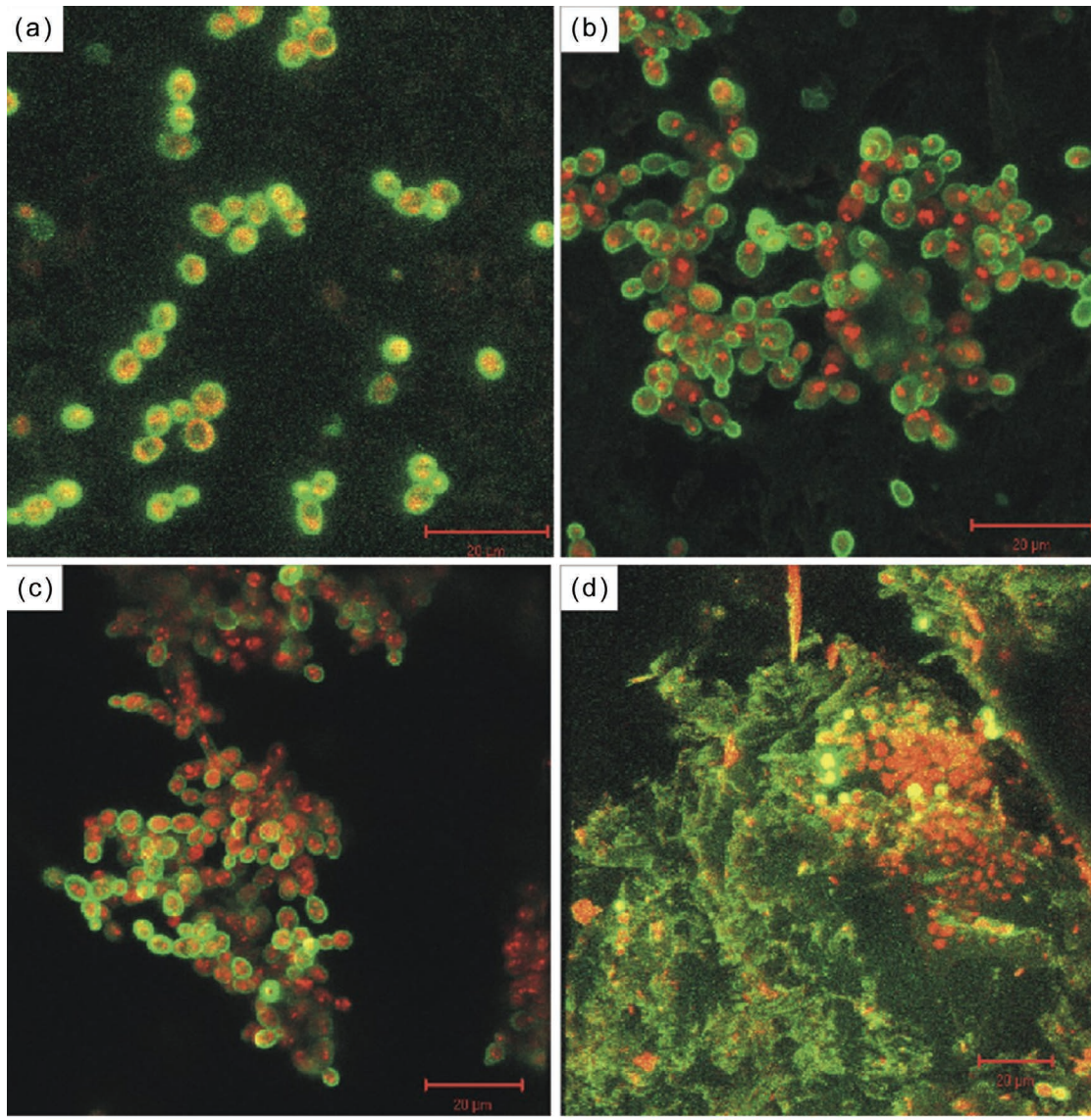
antimicrobial medications as well as attacks by the host immune armamentarium, manifesting in various physiologic problems for humans, as summarized in Table 2.

These biofilm-associated diseases are largely due to the extensive use of IMDs including catheters, prosthetic heart valves, pacemakers, implants, cerebrospinal fluid shunts, among others.[5,19]

While there are several human diseases associated with biofilm production, there are certain conditions that specifically concern the pediatric population. The development of otitis media and acute otitis media caused by biofilms has been extensively studied in the pediatric population, where biofilm formation is naturally more likely to occur owing to the Eustachian tube being shorter and wider in children than in adults, allowing for bacteria such as *S pneumoniae* and *Haemophilus influenzae* to spread rapidly.[20,21] Tympanostomy tube insertion is also a common procedure for children and is used to treat this condition with effusion. Here otorrhea is a common complication that can lead to biofilm growth, and several studies have demonstrated that failure to control the biofilm growth can lead to tube removal.[19,22]

Pertussis (also commonly referred to as whooping cough), is associated with *Bordetella pertussis* as well as *B parapertussis*. This disease—although easily avoided by vaccination—has been on the rise in children.[19] Biofilm growth in relation to pertussis has been studied in mouse models, where growth

Figure 3B. Confocal scanning electron microscopy examination showed that *Candida* biofilms pass through (a) adhesion phase (2 hours), (b) proliferation phase (8 hours), (c) microcolony formation (8 hours), and (d) maturation phase (24–48 hours).[16]



on the ciliated epithelium was observed, and a link between increased biofilm production and infection was observed. It has also been suggested that biofilm growth itself can be a contributing factor to pertussis. Thus, it was suggested that one way to control biofilm formed in pertussis is to include biofilm protein antigens in pertussis vaccines.[19]

Urinary tract infections can be a chronic condition, and a growing amount of evidence points to the intracellular bacterial community (IBC) as the root cause of its persistence. In mouse models, it was found that IBC growth is facilitated by the formation of biofilms, which allows the bacteria to grow with minimal disruptions. In the pediatric context, IBC/biofilm growth was found in around 36.8% of children with cystitis,[23] indicating a significant occurrence of biofilm-related problems within the pediatric population as well.

Another risk factor for developing biofilm-associated infections in the pediatric population is the extensive use of parenteral lipid emulsion (LE).[24] Our team studied the effect of LE on the ability of *Candida* to germinate and form biofilms on medical catheter material.[25] Our testing showed that adding LE to standard fungal growth medium increased the ability of *C albicans* to form biofilms and led to changes in biofilm architecture and morphology. Moreover, incorporation of LE

Table 2. Biofilm-Associated Diseases and Targeted Organs

Body System	Affected Organs	Disease
Auditory	Middle ear	Otitis media
Cardiovascular	Cardiac valves Arteries	Infective endocarditis Atherosclerosis
Digestive	Salivary glands	Sialolithiasis
	Gallbladder	Recalcitrant typhoid fever and predisposition to hepatobiliary cancers
	GI tract (especially the small and large intestine)	Inflammatory bowel disease and colorectal cancer
Integumentary	Skin and underlying tissue	Wound infections
Reproductive	Vagina Uterus and fallopian tubes Mammary glands	Bacterial vaginosis Chronic endometriosis Mastitis
Respiratory	Nasal cavity and paranasal sinuses Throat (pharynx with tonsils and adenoids, and larynx with vocal cords) Upper and lower airways Upper and lower airways	Chronic rhinosinusitis Pharyngitis and laryngitis Pertussis and other Bordertella infections Cystic fibrosis
Urinary	Prostate gland Urethra, bladder, ureters, kidney	Chronic bacterial prostatitis Urinary tract infection

GI, Gastrointestinal

to the growth media induced candidal germination (a critical virulence factor for *C albicans*). Our results provided insight into the underlying mechanism for the increased risk of candidemia in pediatric patients receiving LE via medical catheters.

Overall, biofilms play a crucial role in the production and persistence of several diseases, especially within the pediatric population. This highlights the ongoing need to test and develop novel approaches to manage and control biofilm-related human diseases in this population.

Challenges and Treatment Strategies

Biofilm Management. There are 2 main management strategies when it comes to combating the health effects of biofilms: prevention or treatment (a responsive measure, taken after the biofilm has matured). Both routes have several challenges, as highlighted in Table 3.

Preventative Measures and Challenges. Preventative measures focus on interrupting biofilm growth and production well before the patient manifests symptoms of biofilm-related diseases. A perfect time to block the development of biofilms is interrupting the adhesion phase, thereby interfering with matrix formation. Inhibiting the ability of the microorganism to adhere *in vivo* or to inanimate surfaces offers a potential solution to the problem. This can be achieved by using a special surface coating (which is created by growing nano-daggers) to further prevent the adhesion of the microbial cells and decrease the chance of biofilm creation.[15] A key challenge in such strategies, however, is the resulting dead cell mass and debris; however, the nano-dagger method effectively controls for this by also inhibiting the ability for these masses to coagulate. Several additional options for decreasing bacterial adhesion are available (such as gold nanoparticle layer–phase transition lysozyme film coating, zwitterionic hydrogel coating, among others), however these techniques are increasingly complicated and may not be practical in many settings. These coating types (because they are created as a mixture of 2 coatings) may also be unable to retain their capabilities in the long run.[26] In the medical context, it is important to understand the complexity behind implanting different coatings in certain patients, as there is always a chance of immunologic rejection of the coating. Hence, more research also needs to be done prior to bringing novel coatings to clinical practice, as well as making them adaptable to a variety of environments (e.g., varying temperatures and pressures).

Another approach to inhibiting biofilms recognizes that the biofilm matrix depends on specific proteins for its structure and drug resistance. The Csg A and B proteins are crucial for forming the biofilm, while Lec A and B contribute to its resistance against drugs.[26] By disrupting the function of these proteins, it is possible to significantly delay the development of the biofilm. This is possible by using small-molecule inhibitors to block binding sites of Csg A and B in order to prevent

Table 3. Summary of the Challenges in Both the Prevention and Treatment Methods of Biofilms

Preventative	Treatment
Dead cell mass and debris coagulation can be caused by the methods used to destroy the biofilm	Antimicrobials have very limited efficacy in terms of curing biofilm-related infections due to resistance to treatment
Complexity of coating techniques may not make all treatments feasible for the general population	Antibiotic resistance further complicates the ability to cure matured biofilms
Long-term efficacy needs to be established for several methods	Photothermal therapy can risk other healthy cells owing to the high heat required
Immunologic rejection is a risk as coatings may be rejected by the patient's body	As with preventative measures, treatments also have immunologic risks
Interfering with bacterial metabolism is relatively novel and needs further development	Nanometers as a treatment is still in its infancy and needs further research

polymerization and assembly of biofilms. RNA interference can also be used effectively to reduce the expression levels of Lec A and B, thus diminishing their role in drug resistance.

Another route that can be taken to prevent biofilm formation is by interfering with the signaling between bacteria that is primarily implemented through the use of molecules termed *autoinducers*. Autoinducers allow for cell-density–dependent regulation of expression,[26] and the quorum sensing (QS) systems that facilitate bacterial communication hold the key to inhibiting bacterial communication, and therefore biofilm production. The QS system of bacteria, however, is complex to characterize, rendering this method of prevention less realistic to implement in patients in a timely fashion.

The final preventative measure to inhibit biofilm formation is through metabolic interference. Bacteria, in their basic form, are migratory organisms. Through altering the metabolism of bacteria, it is possible to make them lose this migratory capacity, and greatly reduce the chance of biofilm growth. It is currently hypothesized that the alteration of purine biosynthesis and wound-healing metabolic pathways[9] are leading to the growth of biofilms. However, similar to the aforementioned bacterial-signaling method, this method is also novel, and more research is needed to completely harness this approach.

Treatment Measures and Challenges. Treatment measures focus on tackling the biofilm after it has matured. The first strategy often used in response to biofilm production is the use of antimicrobials, although these often have minimal efficacy on decreasing biofilm-related diseases and infections because most biofilms are diagnosed at a late stage, when they are more developed and less likely to be affected by antibiotics. This has caused a rise in the study of antimicrobial peptides, which have been found to help curb the impact caused by antibiotic resistance. Bacteriophages (phages) are another alternative to antibiotics, because they are less disruptive to the patient's system, are more cost-effective, and more targeted. The invasion technique of phages allows for the structure of the biofilm to rapidly deteriorate, therefore allowing faster relief for the patient. This technique, however, is one of the latest efforts to combat biofilms and needs several additional clinical trials as well as a greater understanding of how the human immune system can react to phages (to avoid situations such as allergic reactions or other negative immunologic responses). Reactive oxygen species (ROS) are yet another strategy, as they cause peroxidation reactions that damage the nucleic acids, proteins, and structures that give the biofilm its properties.[15] Delivery methods for ROS include photodynamic therapy, where a photosensitizer generates ROS upon laser activation. Additionally, nano-enzymes like CoPt@graphene (G) @glucose oxidase (GOx) (CoPt@G@GOx) can produce ROS from glucose, targeting infections without relying on oxygen. These systems enable controlled release of ROS at the infection site, enhancing their antibacterial efficacy against biofilms.[15]

Photothermal therapy is another possible choice for biofilm treatment, where light irradiation is used to induce local hyperthermia. The purpose of doing so is to disrupt the nucleic acids and proteins of the biofilm to deactivate them, and ultimately sterilize them. The use of this method poses a challenge, as temperatures above 70°C are used, which can have a detrimental impact on other, healthy cells in the body. Another method by which the biofilm matrix can be destroyed is by using nanomotors, because they possess more motion and greater permeability. Thus, nanomotors can permeate the matrix and effectively allow for secondary drugs to penetrate the biofilm and attack the microbe within. While promising, this technique is still in need of more research to ensure safety.[27]

The final option for treatment is one of the most simple and easy to implement: probiotics. *Enterococcus faecium* and *Pediococcus pentosaceus* are examples of beneficial bacteria with properties that can affect biofilm production.[28] The incorporation of probiotics into a diet can be an effective, low-cost, and straightforward method to avoid the complications of biofilm accumulation. A study focusing on treating biofilms demonstrated that a combination of probiotics and amylase effectively disrupts biofilm structure. Specifically, the probiotic formulation, which includes

Bifidobacterium breve, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Saccharomyces boulardii*, and amylase significantly decreased *Candida* growth within 4 weeks of daily consumption. This suggests that such probiotic-enzyme combinations can be effective in managing gastrointestinal biofilms and improving overall gut health.[29] These methods of destroying the mature biofilm are summarized in Figure 4.

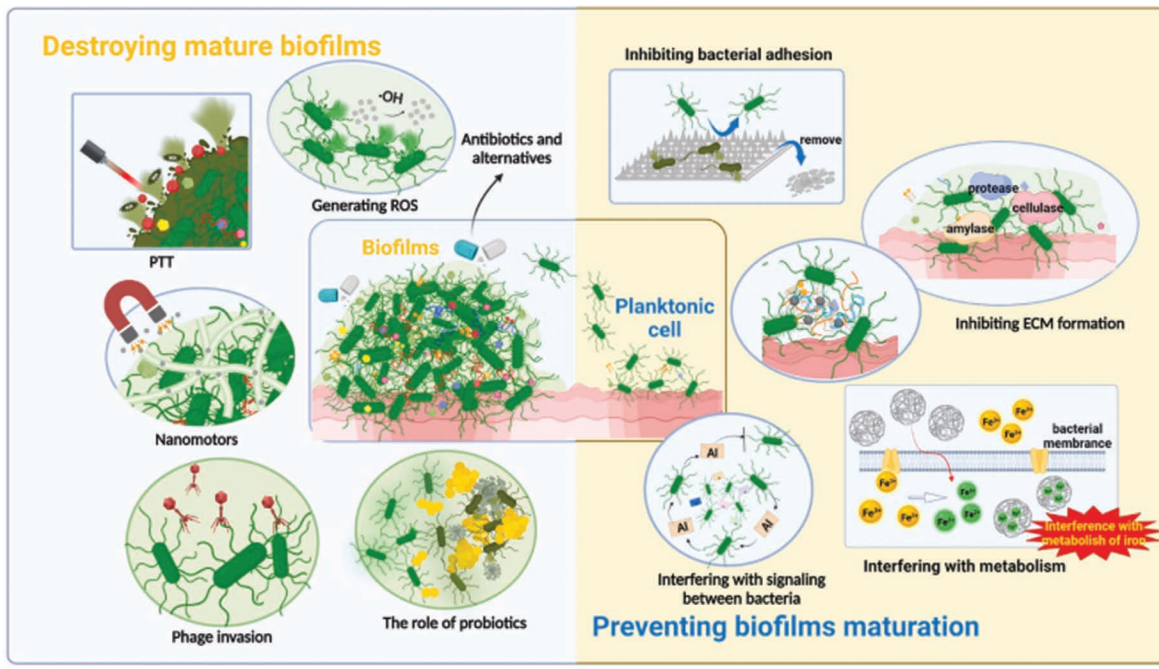
Diagnosis and Treatment Strategies for the Future

It currently remains very challenging to diagnose biofilm-related diseases and infections because they manifest as nonspecific symptoms in patients and there is currently no specific clinical protocol in practice to diagnose such conditions.[30] An example of a clinical case that highlights the complexities of dealing with the condition is presented in Figure 5. In general, a typical biofilm-related infection in a clinical setting presents as a chronic infection that worsens in intervals and that slightly alleviates after antibiotic therapy but does not completely resolve. Traditionally, biofilm growth can be detected through collecting a sample from the patient, performing microbial cultivation, and identifying antibiotic susceptibilities. The device suspected to cause the infection (such as a catheter) can also be removed and taken for further microbial testing.[31]

There are several published techniques detailing how biofilm-related diseases can be diagnosed; however, these methods are often laborious and not practical in the clinical setting. One of the more reliable techniques currently being studied is the use of biopsy to detect biofilm-related disease. This involves obtaining a sample from the patient and staining the sample to visualize the matrix along with other characteristics of the biofilm and immune response.[30] Biopsies are not always indicated in the clinical setting; in these situations, sonication is another promising technique that separates the biofilm (aggregated microbes) from the patient's surface implant and is analyzed.

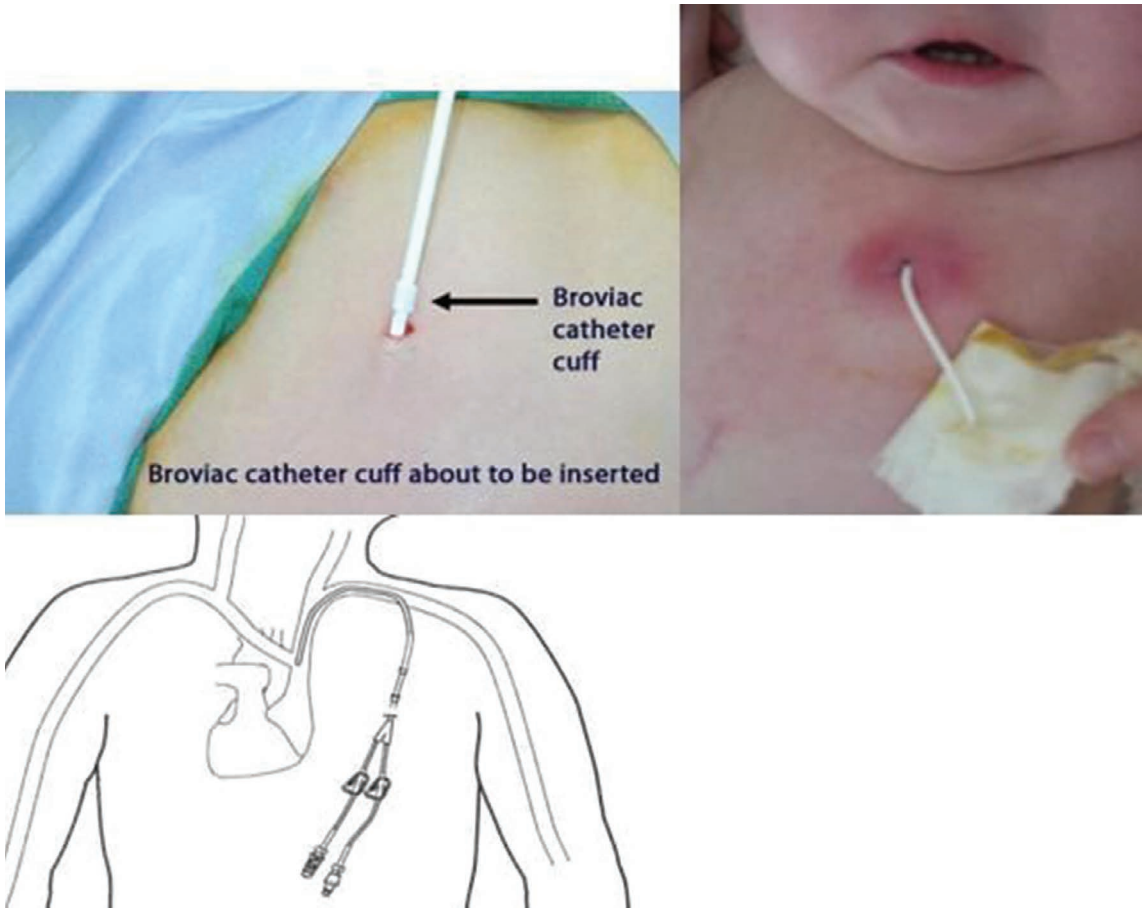
Several novel diagnostic strategies are also being developed, with crystal violet and *Drosophila melanogaster* being among the most promising because of their several advantages. Crystal violet is a low-cost, simple technique, with high reproducibility. It involves staining the entire structure of the biofilm and allows for a total assessment of its biomass. Because the entire structure is stained there is a loss of specificity; the need to also wash the biofilm after being deposited into the plate results in a loss of important biofilm

Figure 4. Schematic representation of reactive and preventative approaches to combating biofilm maturation. The preventative approach strategy focuses on stopping the development of biofilm at different stages of its growth. The reactive approach involves options such as antibiotics, nanomotors, phage invasion, probiotics, and ROS generation.[15]



ECM, extracellular matrix; PTT, photothermal therapy; ROS, reactive oxygen species.

Figure 5. The above is a clinical case published in 2005 in *Pediatric Infectious Diseases*[1]. This serves as a good example of the complexity of handling biofilm production, as it is not always feasible to remove the catheter. After 2 days of treatment, the patient became afebrile, and blood cultures at the end of the antifungal lock period were negative. Antifungal lock therapy with liposomal amphotericin B was initiated and continued for 2 weeks, along with systemic treatment for an additional week, resulting in sterile blood cultures and no signs of deep-seated mycosis. The findings suggest that when central venous catheter removal is not feasible, 8-hour daily antifungal lock therapy combined with systemic administration may be an effective treatment option for managing catheter-related infections[1].



components, highlighting the areas of improvement. *D melanogaster* offers *in vivo* biofilm detection, with high homologies between the *drosophila* and human genomes, is easy to work with, and is inexpensive to operate.[30] Overall, *D melanogaster* is used in biofilm detection by serving as a model organism for studying infections *in vivo*. Researchers have used this approach owing to challenges in mammalian studies, such as ethical approval. Studies have shown that *D melanogaster* can be orally infected with *Vibrio cholerae* to explore biofilm-related behaviors and host interactions. Techniques involve monitoring the effects of QS on the host's metabolic pathways during these infections, which have allowed researchers to gain deeper insights into the role of biofilms in disease progression.[30]

Treatment of a biofilm-associated infection or disease is mainly dependent on whether the biofilm growth is caused by an endogenous or exogenous factor. If a non-foreign body is the cause of infection or disease, high-dose antibiotics given over a long period can significantly reduce the problem. For exogenous causes, removal of the device causing the biofilm buildup will be the fastest and most effective solution.[31]

Conclusion

Looking ahead, it is important to understand that the complexity of treating biofilm-associated diseases can be significantly decreased by detecting the biofilm growth in its early stage. Today's medical technology in the context of biofilms works as a treatment rather

than preventive measure, and with further medical advances, biofilms can be tackled with a preventative focus. The ability to control biofilm growth at an early stage will increase the efficacy of medications (such as antibiotic treatment), will decrease the need to administer various treatments, and will invariably improve the patient's quality of life.

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