







Advances in pulmonary drug delivery targeting microbial biofilms in respiratory diseases

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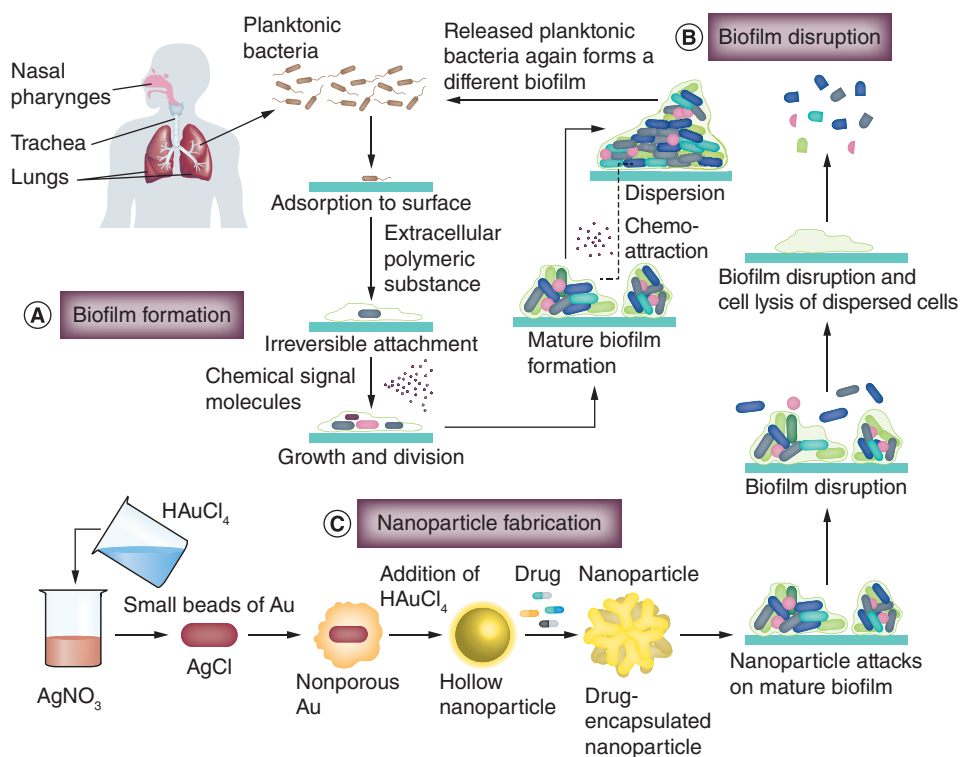
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The increasing burden of respiratory diseases caused by microbial infections poses an immense threat to global health. This review focuses on the various types of biofilms that affect the respiratory system and cause pulmonary infections, specifically bacterial biofilms. The article also sheds light on the current strategies employed for the treatment of such pulmonary infection-causing biofilms. The potential of nanocarriers as an effective treatment modality for pulmonary infections is discussed, along with the challenges faced during treatment and the measures that may be implemented to overcome these. Understanding the primary approaches of treatment against biofilm infection and applications of drug-delivery systems that employ nanoparticle-based approaches in the disruption of biofilms are of utmost interest which may guide scientists to explore the vistas of biofilm research while determining suitable treatment modalities for pulmonary respiratory infections.

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Graphical abstract:



Biofilms are defined as colonies of microorganisms surrounded by self-secreted matrices that grow on living or inert surfaces (Figure 1). Generally, biofilms are deposited on abiotic and biotic surfaces. For instance, they can grow on rocks (abiotic), plants, fungi or a human host (biotic). Biofilms are one of the primary causes of lower respiratory tract infections. These infections may be particularly complicated to treat and are one of the foremost causes of death in developing countries worldwide [1]. Patients with chronic respiratory diseases are at particularly a higher risk of such infections and are one of the principal causes of morbidity and mortality in this group [2]. In addition to the high mortality and morbidity rates, respiratory infections can also impact economies because they can lead to increased treatment costs [3].

The treatment of respiratory infections is challenging because the infections may possess multidrug-resistance, and the microbes may reside deep inside the airways. Delivery of drugs to such deeper parts of the airway system remains a major milestone to be accomplished. Despite the efforts of oral or parenteral administration, the concentration of drug that reaches the site of the problem is small and therefore, high doses of the active drug are needed to maintain the level of drug greater than their minimum inhibitory concentrations (MIC) at the site of infection [4]. Unfortunately, administration of high doses of certain drugs may result in increased toxicity and can even lead to acute neurotoxicity and nephrotoxicity in the patients [5].

Controlled discharge of drugs or antibiotics to the respiratory tract might be an effective solution to this problem because discharge of target drugs or antibiotics directly at the point of infection would allow a higher concentration of drug to reach the targeted biofilm with low systemic exposure [6]. Such a result was seen in a pharmacokinetic study that involved nebulization of the drug colistin methanesulfonate (2 million IU) in cystic fibrosis (CF) patients. The findings revealed that nebulization allowed high concentrations of the drug to be accumulated in the sputum (C_{\max} 6 mg/l), which could be maintained above MIC₉₀ for up to 12 h with negligible systemic drug exposure (C_{\max} <0.3 mg/l) [7].

Research in biofilms with respect to respiratory diseases has not been explored fully and therefore requires attention to maximize the effectiveness of the drugs in use and to improve the treatment methods and strategies available.

This review focuses on the various treatment approaches, such as nanocarriers, systemic therapy and inhaled formulations, that can be used to tackle the biofilms causing respiratory infections. The approaches are discussed in detail with attention to their potential benefits and drawbacks along with possible future directions.

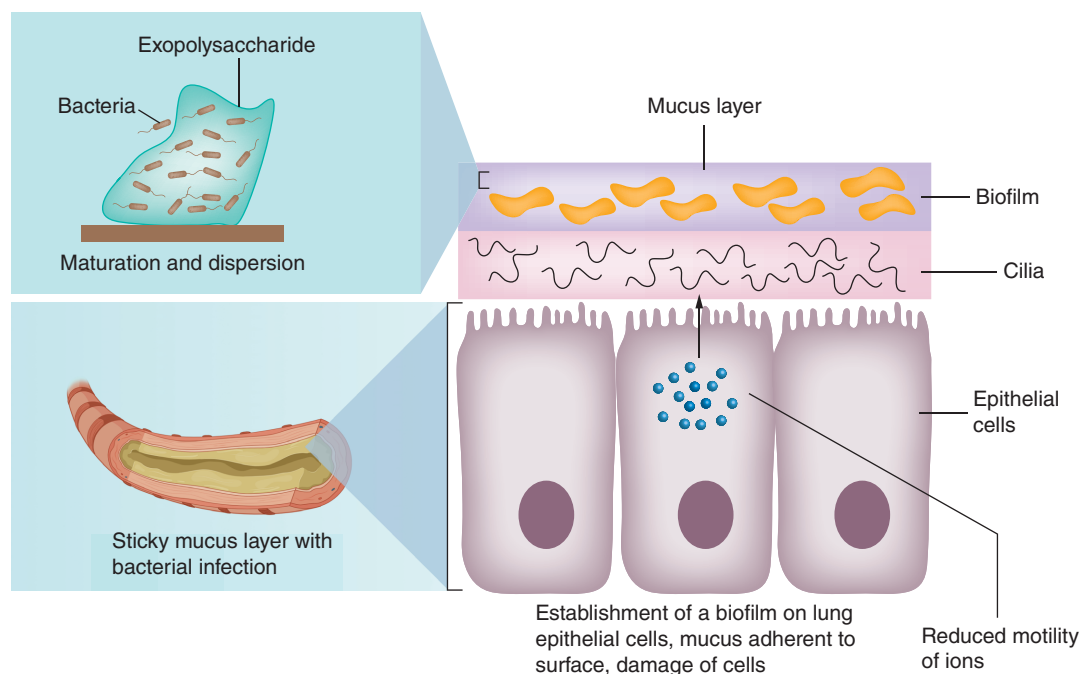


Figure 1. Schematic representation of cystic fibrosis lung epithelial cells. The epithelial cells of a cystic fibrosis lung are covered by a thick, sticky mucus including bacterial biofilms, leading to a block of cellular activities and breathing problems.

Biofilms: formation, multidrug-resistant properties & prevention

Intensive studies have been conducted in the past 2 decades to investigate microbial development in biofilms, especially, in biofilms that are found in the natural aquatic system predominantly attached to the surface of micro-aggregates or macro-aggregates.

Biofilm development and maturation have been demonstrated and observed in a variety of bacterial species, including *Escherichia coli*, *Pseudomonas aeruginosa* and *Vibrio cholera* [8–10]. In such studies, experiments were conducted using a genetic screen where randomly assorted transposon mutants were allowed to mature in a 96-well plate [11]. The findings of the studies show that the bacterial colonies primarily employ either their flagellum or type IV pili for movement along surfaces in a collective motion until they interact with other bacteria, resulting in the formation of small colonies, where they eventually expand and mature [8]. The production of exopolysaccharides (EPS) helps the bacteria to become more stabilized [9]. Studies have also revealed that wild-type *V. cholera* has a specialized structure that provides a greater advantage to the biofilms in providing them the ability to form colonies on the surface. It is believed that the rate of adherence might be the key factor in obtaining a place to reside in the microbial colony.

Biofilms may constitute either a sole species of microbe or several microbial species. Biofilms of a single species are those generally found on the exteriors of the medical implants and in a wide range of infections in humans [12]. One of the key areas of research today involves single species biofilms that are capable of causing various respiratory infections.

Biofilms incorporate an assemblage of microorganisms (bacteria or fungi) that are integrated into a unit containing EPS, proteins and nucleic acids. Bacteria or fungi that can form biofilms are possibly morphic and can germinate on surfaces such as medical devices, endotracheal tubes and tracheostomy tubes [13,14]. It has been observed that biofilms are dependent on the fabricated material of such devices [15]. Biofilms cause serious diseases and are a cause of worry for patients with severe respiratory diseases like CF and chronic obstructive pulmonary disease (COPD) [16].

Multidrug-resistant properties

The multidrug-resistant properties of biofilms against antimicrobial agents may be due to several factors that are discussed here in brief. One of the main reasons is the failure of antimicrobial drugs to penetrate the biofilm barrier.

This also poses a challenge to drug delivery. A commonly found and used disinfectant, chlorine, was reportedly used against *Klebsiella pneumoniae* and *P. aeruginosa* biofilms. The observations were measured with a chlorine-detecting microelectrode. The results showed that not even >20% of chlorine reached the bulk media's concentration [17]. The EPS matrix may also play a key role, which may contribute toward the delay in the penetration of antimicrobial drugs. However, EPS is not the only factor that contributes to the multiresistant nature of the biofilms. There are other multiple mechanisms such as slow growth rate, stress response and heterogeneity.

The bacterial cell culture shows a period of slow or no growth during limited supply of nutrients. This transition to slow or no growth is suggested to be one of the physiological mechanisms that may lead to antibiotic resistance [18]. This characteristic feature is usually seen in mature biofilms [18,19]. This phenomenon was also reported in *P. aeruginosa*, *E. coli* and *Staphylococcus epidermidis* biofilms under limited growth conditions by Ciofu et al. [20]. A generalized observation was made that the effect of either tobramycin or ciprofloxacin had increased along the growth rate in both planktonic and biofilm cells. Therefore, it was suggested that the slow growth rate of biofilm cells was a protective mechanism against antimicrobial agents.

Lastly, heterogeneity contributes to the resistance against antimicrobial agents. The activity of *K. pneumoniae* biofilm, when treated with monochloramine, showed that the biofilm cells that were closest to the biofilm-bulk-liquid interface perished first [21]. The study further revealed that the activity of antimicrobial agents may vary depending on the location of cells inside a biofilm.

Prevention of biofilm

Biofilm-associated infections occur frequently through skin-habitant bacterial organisms such as *Staphylococcus* spp. and opportunistic fungi such as *Candida* spp., which are seen in immunocompromised patients. Biofilms have the ability to grow on abiotic surfaces, which may serve as a virulence factor for microbial biofilms [22].

Therefore, strategies to prevent these infections focus on surface coating with antimicrobials and antiseptics. This would also ensure no microbial adhesion and prevents biofilm formation. To combat the surface adhering biofilm formation, the development of novel biomaterials such as metal nanoparticles (NPs) with surfaces modified to alter the biophysical cell-surface interaction are favored because this prevents growth of biofilm.

Another approach to prevent biofilm formation focuses on disrupting the signaling pathways of bacterial cell that coordinate with the genes responsible for biofilm development. For instance, the (p)ppGpp, cyclic-di-GMP inhibitors and quorum sensing (QS) signaling have shown positive results in the prevention of biofilm formation and could also potentially lead to novel therapeutic strategies [23].

Various types of biofilms in respiratory infection

Biofilms exhibit taxonomic diversity in many microbes, such as bacteria and fungi that cause infections in the respiratory system. Some major biofilm infection organisms include *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp., *P. aeruginosa*, *Candida* spp. and *Aspergillus fumigatus*. *P. aeruginosa* and *A. fumigatus* are mainly associated with pulmonary disease, which are further discussed. There are researchers considering *Mycobacterium abscessus* and *Haemophilus influenzae* as a potential pathogen for biofilm formation.

H. influenzae has been reported to possess the ability to express neuraminidases that cleave α 2,3-linked sialic acids from glycoconjugates. Neuraminidases (sialidases) are generated to adhere to the host cells by various kinds of mucosal pathogens as seen in *Streptococcus pneumoniae* in the airway and *V. cholerae* in the gut [24]. The function of neuraminidase is firmly established in influenza pathogenesis, whereby it helps in designing a treatment method against it. However, the role of bacterial neuraminidase in the pathogenesis of disease is not fully understood yet. *P. aeruginosa* comes from neuraminidase-secreting species that colonize the highly sialylated discharge and the surface of upper respiratory tract [25]. The aforementioned neuraminidase secreting species may bind to the asialylated glycolipids produced by neuraminidase action. However, this species shows varying metabolic activity or differs in infusing sialic acid to its surface structures [26]. Therefore, it is highly possible that bacterial neuraminidase engages with both microbic and eukaryotic glycoconjugates [25].

Mycobacterium spp. have also demonstrated their ability to form biofilm, most notably *Mycobacterium tuberculosis*, which assembles itself into a highly organized matrix-encapsulated biofilm complex under favorable conditions [27]. Other nontuberculosis mycobacteria (NTM), *Mycobacterium avium* complex (MAC), also the fast-growing *Mycobacterium abscessus* complex (MABSC), were observed to grow biofilms *in vitro* and environmental reservoirs [28]. MABSC has emerged as a threat to CF patients who suffer from it at an early age and deteriorate clinically as the consistent infection results in tissue damage and inflammation [28,29]. There is a lack of direct evidence that

suggests that MABSC is responsible for pulmonary biofilm infection, but indirect evidence supports this claim. Laboratory and environment cultures have observed the growth of biofilm by NTM. The sputum of NTM patients has also shown biofilm like aggregates in the acid-fast smears of sputum. *Mycobacterium ulcerans* is responsible for chronic ulcers. The microbe lives in a tropical environment and is also a member of NTM, which indicates biofilm infection [29]. Biofilm infections are found on surfaces but also penetrate deep into tissues as previously shown in chronic wounds [30]. Similarly, MABSC might be capable of forming biofilm infections in the human lungs. Biofilm growth could also explain the failure of therapeutics in MABSC infection and its susceptibility testing. A study indicated two possible explanations. First, the diffusion of antibiotics was inhibited by the biofilm due to the presence of an antibiotic concentration gradient across the biofilm aggregates leaving the bacteria viable [31]. The second explanation suggests that it might be due to the heterogeneity of metabolic state present in a biofilm that is able to shield the dormant bacteria and also allow for an adaptive evolution of the consistent bacteria [32]. The MABSC biofilm infection needs more research and requires discovering more in-depth evidence that could also lead to the discovery of more biofilm-disrupting agents.

Another prominent biofilm forming pathogen is *A. fumigatus*, which is responsible for causing severe pulmonary infections. *A. fumigatus* is a ubiquitous filamentous fungus whose pathogenesis is beginning to emerge. This has helped scientists to understand the importance of biofilm formation during an infection. *A. fumigatus* is known to invade the respiratory systems of immunocompromised patients. The organism also inhabits the airways of CF patients and patients suffering from other chronic pulmonary diseases. The results of histopathologic studies conducted on human tissues and animal models of invasive chronic pulmonary infection have revealed that *A. fumigatus* grows as an aggregation of hyphae within an extracellular matrix embedded as seen in a biofilm [2,33]. Experimental studies have shown that the biofilms have contributed to the virulence of this fungal species by promoting enhanced adherence to the host cell [34]. In addition, it may increase resistance to antifungal agents and host immune system. The biofilm formation by *A. fumigatus* in pulmonary diseases has resulted in a higher failure rate of antifungal therapies in the treatment of invasive aspergillosis. Limited studies have uncovered the full potential of *A. fumigatus* infection, which warrants more in-depth preclinical studies to understand the invasive nature of the organism and its long-term consequences.

Diagnosis of biofilms

Biofilms do not have definitive diagnostic markers. This makes their recognition and diagnosis difficult. Biofilms are classified on the basis of a combination of microbial groups, clinical and microscopic features including both localized and persistent infections, or direct examination of infected tissues to detect microbial biofilms [35]. Biofilm growth can also hinder the microbiological diagnosis because the microbes could not be recovered for culture as that would cause physical disruption of surface attached biofilms.

Moreover, the *in vitro* antimicrobial susceptibility test has restricted percentage value in anticipating the activity against biofilm infection. Although the antimicrobial susceptibility assay tests are somewhat useful when tested for microbes in the planktonic state. With constant change and improvement in technology the antimicrobial susceptibility test assay for biofilms have been made available. However, they still are not as reliable for predicting microbiological or clinical outcomes when assessed in patients with CF infected with *P. aeruginosa* [36]. Although there have been some developments for bacterial biofilms, there have been limited advancements in the area of fungal antimicrobial susceptibility testing.

Challenges in biofilm treatment

The conventional antibiofilm drugs used in treatment strategies to date have restricted therapeutic activity against biofilm infection.

There exist several challenges reported in the treatment methods, which include the following: identification of a suitable antimicrobial compound, selection of a suitable drug-delivery device and optimizing the formulation for efficient target drug delivery, especially in the sinuses and nasal cavity [37].

One of the strategies involved the use of a powder, which comprises amorphous colistin and crystalline rifampentine with an extended antimicrobial activity against planktonic cells and *P. aeruginosa* biofilm employed for respiratory infections [38]. In another study, amphibian AMP esculentin, which was employed as a new antibiotic formulation, demonstrated significant effects in controlling the growth of *P. aeruginosa* in sepsis and pulmonary infections [39]. This may be potentially employed as a drug to disrupt the biofilms. The findings indicate that esculentin prolonged the survival of animals both in sepsis and during pulmonary infections.

Table 1. Major inhaled formulation against biofilm infection.

Serial number	Biofilm-associated infection	Major organism	Target drug
1	Cystic fibrosis	<i>Pseudomonas aeruginosa</i>	Aztereonam
2	Pulmonary infection/disease	<i>Aspergillus fumigatus</i>	Alginate combined with apolytene antifungal
3	Nosocomial	<i>Staphylococcus epidemidis</i>	Cefazolin and vancomycin, gentamicin neutralized with rifampicin

Several recent research advancements have suggested the successful modification and utilization of antimicrobial transport mechanisms that are reported to be effective against several important microbes that reside in the biofilms. Moreover, diverse NP-drug delivery and photodynamic approaches have been reported to effectively target and disrupt biofilms by forming potentially lethal reactive oxygen species [40,41]. It is well known that photodynamic modifications seem relatively common in microbes observed in the oral cavity and skin [41].

Antimicrobial tolerance of biofilms *in vitro* & *in vivo*

Tolerance *in vitro*

The antimicrobial tolerance of biofilms is believed to be linked to the growth mode of the biofilm. If the bacteria have a planktonic culture, it will exhibit susceptibility to antimicrobial activities [18]. Tolerance in a biosystem is multifactorial and is fundamentally different from antimicrobial resistance. It is rather attributed to restricted penetration of antibiotics, limited growth, low oxygen levels, expression of biofilm-specific genes and presence of persisters.

Restricted penetration

As discussed in the preceding sections, biofilms restrict the entry of antimicrobial agents and do not permit the diffusion of drugs. The restricted penetration may occur when the antibiotics bind to the components of the biofilm matrix or bacterial membrane, such as the extracellular DNA [19,42,43].

Differential physiological activity

Differential physiological activity of microbes in biofilm is another cause of concern in antimicrobial tolerance. The metabolic activity is high on the outer regions and low in the inner regions of biofilms. This is due to the limited oxygen and nutrient penetration as seen in *P. aeruginosa* [20].

Differential expression of specific genes in biofilms

Biofilms can show that a specific expression of genes may also contribute to a specific tolerance mechanism. For instance, *ndvB* gene (*PA14*) in *P. aeruginosa* encodes for an enzyme that synthesizes periplasmic glucans binds to tobramycin. This results in the prevention of cell death by isolating the antibiotic.

Persister cells

Persister cells are considered to be an outcome of the bacterial differentiation into a dormant stage. They can decrease the metabolic rate, which evidently enables them to escape the effect of antibiotics even to those that kill nongrowing cells [44,45].

Tolerance *in vivo*

The sputum obtained from patients with CF and chronic *P. aeruginosa* infection contains polymorphonuclear leukocytes that get assembled at the site of biofilm infection and intake the oxygen, thereby creating an anaerobic condition, and limiting bacterial growth [46]. *In vitro* models explain the restricted growth in the deeper layer within a biofilm, but in such models, the biofilm itself may be located in the oxygen-deprived areas as a result of polymorphonuclear leukocytes (PMN) inflammation or physiological conditions. The CF sputum and sinus secretion of CF patients were also seen reporting lower oxygen tensions during biofilm infections [46,47].

Current treatment methods

Antibiotic treatment (high concentrations) administered through a topical route

Topical administration allows the delivery of antibiotics at a high local concentration directly to the target site effectively avoiding side effects and undetectable serum concentrations. In one study, nebulized tobramycin was

found to be 1200 mg/l in the sputum of the patient when only 1 mg/l was found in the serum concentration [42]. The current antibiotic regimen used in biofilm-originated lung infection in CF is as follows:

- a) Tobramycin (300 mg) through inhalation;
- b) Aztreonam (75 mg) through inhalation;
- c) Ciprofloxacin (65 mg) through inhalation;
- d) Levofloxacin (240 mg) through inhalation.

The antibiotics that are employed in biofilm-caused lung infection in non-CF bronchiectasis are as follows:

- a) Colistin (1 MU) through inhalation;
- b) Tobramycin (300 mg) through inhalation;
- c) Ciprofloxacin (32.5 mg) through inhalation.

The nebulized antibiotic administration of antibiotics through inhalation have shown a marked improvement in pulmonary symptoms and have minimized the bacterial load in the sputum. The formulations were also reported to be well tolerated [48].

Combined & sequential antimicrobial therapies

Antimicrobial drugs are used in combination, especially in patients with CF [49]. This may be primarily done to prevent the maturation and multiplication of multidrug-resistant biofilms. Biofilms are capable of having varied structural functions and different metabolic states. Therefore, combining ciprofloxacin, which attacks metabolically active layers, and colistin, which disrupts and kills the biofilm cells during low or inactive metabolic state, may render high therapeutic efficiency [50]. Several *in vivo* and *in vitro* studies have reported combinations that have been proved to be efficient in the treatment of biofilm-driven infections [50,51].

Inhaled antimicrobial formulations: treatment of biofilm-induced respiratory infections

Respiratory infections due to biofilms pose a serious adverse health scenario globally. Bacterial and fungal biofilm-infection agents seem to play a significant role in causing respiratory illnesses today [2,52]. The need to develop inhaled antimicrobial formulations has become urgent because higher doses of systemic therapies have the potential for harmful effects. Inhaled drug formulations both for the treatment and prevention of respiratory infections have become momentous. Recent advancements have encouraged such inhaled formulations for medical therapies. Drug formulations not only target the primary infected sites but also shrink the risk of toxicity to a significant level compared with drugs in systemic therapy. In addition, they also have become a major convenience to work with clinically. The inhaled-drug formulations, as mentioned in [Table 1](#), for the treatment of infections can be classified on the basis of biofilm-led infections. For biofilms of bacterial, viral and fungal origin, the inhaled drug formulations are inhaled antibiotics and inhaled antifungal agents.

Inhaled antibiotics

Inhaled antibiotics or antibacterial agents are used against bacterial-biofilm infections [53]. These antibiotics may be approved for the treatment against CF and similar conditions. These may also be employed for off-label use in non-CF bronchitis hyperventilation-associated pneumonia (VAP) [54]. Using these inhaled antibiotics not only minimizes systemic exposure but also delivers a higher concentrated dosage to the respiratory tract [55]. Three of the approved antibiotics are mentioned in [Table 2](#)

Inhaled anti-fungal agents

In addition to bacterial and viral biofilm infections, fungal biofilm infections caused by *Aspergillus* in the respiratory tract are also one of the major causes of casualties. This usually results from the inhalation of fungal spores. Patients who are already suffering from HIV-AIDS, cancer or organ transplantation are more susceptible to fungal respiratory infections. The global morbidity and mortality rates caused by fungal biofilms are 40–90% [56]. Antifungal formulations are hence important because they not only provide a better alternative against systemic administration of antifungal agents but also considerably reduce systemic toxicity. There are only a handful of inhaled antifungal drugs ([Table 2](#)).

QS inhibitors & anti-QS peptides to control biofilm infections

A recent analysis used synthetic molecules to inhibit *P. aeruginosa* QS receptors, LasR and RhIR [57]. Meta-bromothiolactone used in the study depicted a significant inhibition of production of pyocyanin, a virulence factor and thus prevented biofilm formation [58]. A combination of QS inhibitor FS3 and daptomycin has been used against prosthesis biofilm in a rat model of staphylococcal vascular graft infection [59]. The use of FS3 and daptomycin together was successful in displaying a synergistic efficacy compared with a single mode of treatment.

RNA-III inhibiting peptide was used in a study to restrain staphylococcal TRAP/agr systems and to reduce biofilm infection *in vivo*. The findings point to the importance of quorum sensing in biofilm infection in the host. The use of RNA-III peptide in rats to inhibit methicillin-resistant *S. aureus* graft infections, suggests that RNA-III peptide could possibly be an antibiofilm agent [60].

Antagonists of diguanylate cyclase

Diguanylate cyclase (DGC) antagonists were found to demonstrate impairments on *P. aeruginosa* and *Acinetobacter baumannii* [61]. Ebselen and ebselen oxide are inhibitors of c-di-GMP through inhibition of DGC's limited c-di-GMP in regulation of *P. aeruginosa* biofilm [62].

Bacteriophage therapies

Bacteriophage therapy for bacterial infections has been used in practice for a long time. The development of multiresistant bacteria and antibiotic resistance has drawn attention to bacteriophage therapy [63]. Bacteriophages are able to infect and kill antibiotic sensitive and resistant bacteria.

NP drug-delivery formulations

The currently available antibiotic-delivery systems have failed to eliminate biofilm-protected bacteria. Therefore, there is an urgent need for novel therapeutic approaches, which may effectively target the site of action with minimal side effects and low toxicity. NPs may play a key role to overcome this problem. Several nanoparticles such as PLGA nanoparticles, liposome nanoparticles and chitosan NPs are currently found to be beneficial. The process of biofilm formation and its action of NPs is shown in Figure 2.

Continued interest in NPs as a diagnostic, treatment and drug-delivery tool has been attributed to their adaptability. In the case of biofilm-induced respiratory diseases and infection, nanotechnology serves as potential therapeutic mode to ameliorate concerns and barriers that make biofilms difficult to treat. These include the poor antibiotic penetration and nonspecificity of other administration methods. NP-based treatments includes the use of NPs exclusively or in combined therapies.

Successful delivery strategies have employed lipid and polymeric NP-based formulations such as liposome and cyclodextrins, respectively. Furthermore, inorganic carriers – namely, metal NP such as silver NPs – have shown innate antimicrobial properties. The current NP-based drug-delivery systems is summarized in Table 3.

Lipid-delivery systems

Liposomes

A liposome is a simple structured NP that comprises of a spherical shape and has a lipid bilayer comprising of phospholipids along with sterols, glycolipids, membrane proteins and hydrophilic polymers [66]. The principal application of liposome NP-based formulations is target-specific delivery, which can encapsulate hydrophilic and hydrophobic drug material [67]. This would allow the liposomes to be used in a diverse range of pharmaceutical

Table 2. Major pathogens causing biofilm infection and the drugs in use.

Inhaled formulations	Biofilm pathogen
1. Inhaled antibiotics	
a) Streptomycin	<i>Staphylococcus</i> spp. and <i>Pseudomonas aeruginosa</i>
b) Aztreonam	<i>P. aeruginosa</i> , <i>Acinetobacter baumannii</i> and <i>Stenitrophomonas maltophilia</i>
c) Colistin	<i>Pseudomonas</i> , <i>Acinetobacter</i> , <i>Klebsiella</i> and <i>Enterobacter</i>
2. Inhaled antifungals	
a) Pentamidine	<i>Pneumocystis carinii</i>
b) Amphotericin B	<i>Aspergillus fumigatus</i>

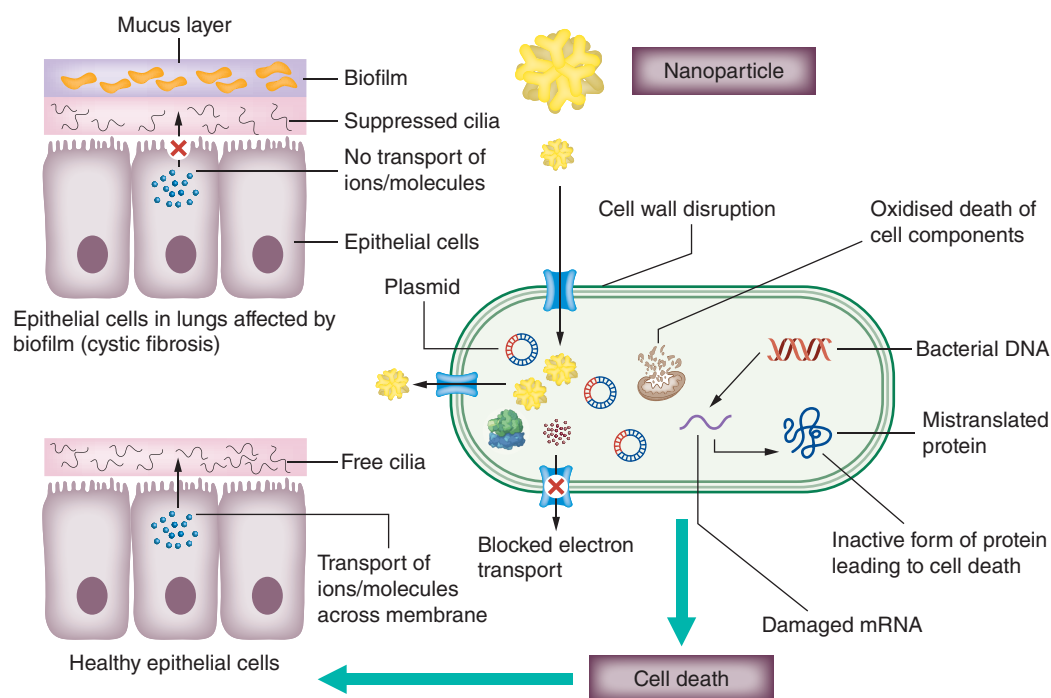


Figure 2. Diagrammatic representation of the potential mechanisms of nanoparticles against biofilm and the treatment of cystic fibrosis lung cells.

applications. Liposome encapsulation also exhibits potential properties of NPs in a restrained-release delivery mechanism that forms an efficient method of drug delivery [68].

A variety of liposome-based NPs have been formulated for delivering potential antimicrobial drugs to the biofilms and biofilm-related microbes. Various studies have revealed the applications of NPs in the drug delivery for biofilm-associated infections. Furthermore, Scriboni *et al.* reported the interactions of liposome-based NPs and some modified liposomes in an *in vitro* of *S. aureus* biofilm [69]. *Streptococcus salivarius* and *Streptococcus sanguis* were studied by Robinson *et al.* in 2001 to observe the variations in absorption between anionic and cationic liposomes in oral biofilms [70]. In the preceding studies reported on biofilms, the emphasis was on the association between the composition of liposomal-NPs, adhesion behavior and the particularized makeup of the biofilms. Adsorption to the biofilm is characteristic to liposomes. The varying compositions of cationic liposomes such as dimyristoyl phosphatidylcholine, cholesterol and dimethyl dioctadecyl ammonium bromide in the ratio of 78.6:23.2:0 to 58.5:23.0:18.5 molar ratio, and anionic liposomes (made of dimethyl dioctadecyl liposome and phosphatidylinositol in 82.5:17.5 molar ratio) show the principal differences in the adsorption behavior with varying compositions. In addition, the significance of electrostatic interactions is studied through the delivery of antimicrobial agents such as nitric oxide. The interactions of cationic and anionic liposomes differ considerably. For a cationic liposome, the bacterial interactions are directed by Van der Waals (electrostatic interaction) forces, whereas, the adsorption of anionic liposomes onto a biofilm is an outcome of an equilibrium between attractive hydroxy (OH) interactions and repulsive electrostatic (Van der Waals) forces associated with H-bonding. The results of antimicrobial efficacy studies show that there are no accumulative outcomes associated with the usage of antimicrobial-liposome particles. However, antimicrobials may damage the action of zinc citrate particles, although the benefit of using the solid supported liposomal system gives an increased stability to the drug encapsulated liposomes. *P. aeruginosa* biofilm have been primarily seen in CF, and a range of formulations with liposome drug delivery *in vitro* have been developed to address this health problem [71].

Solid lipid NPs

Solid lipid NPs (SLN) are made up from solidified lipids, such as triglycerides, fatty acids, waxes and emulsifiers that include lecithin, poloxamers, bile salts and water. SLNs are submicron dispersions of the solidified lipids into a liquid media. SLNs are utilized for the delivery of hydrophilic and lipophilic antibiotics along with some minute

Table 3. Nanoparticle drug-delivery systems.					
	NPs	Mode of preparation	Size (nm)	Applications	Ref.
Lipid-delivery system	1. Liposomes	Thin film rehydration method	50–1000	Widely used for the delivery of antimicrobials to biofilms and biofilm-associated microorganisms	
	2. SLNs	Lipid film hydration method	80–300	SLN formulations of antimicrobial agents is primarily used to target biofilm-forming microorganisms instead of mature biofilms themselves	
	3. Microemulsions	Water-in-oil or oil-in-water microemulsions	10–300	Certain tendencies of microemulsions (e.g., thermodynamic stability, ability to reduce toxicity of encapsulated materials and hydrophilic, hydrophobic, amphiphilic drug solubilizing capacity) make them amenable for controlled release of antimicrobial delivery applications for transdermal, topical, ocular and parenteral administration	[64]
Inorganic delivery system	1. Gold	Chemical synthesis	1–150	It can provide stable, nontoxic, biocompatible delivery vehicles for various therapeutic molecules	
	2. Silica	Sol-gel process	200	Biomedical applications include drug delivery and theranostics	
	3. Silver	Evaporation–condensation or electrical irradiation	1–100	Important in the treatment of biofilm-forming microorganisms due to its antimicrobial property, which includes effects against Gram-positive and Gram-negative bacteria as well as fungi and protozoa Pulmonary infection/disease	
	4. Zinc	Prepared by the sol-gel method, electrochemical depositions, ultrasound and other processes	30	Due to its antimicrobial activity, it acts against a range of oral pathogens, Gram-positive and Gram-negative bacteria and fungal microorganisms; along with zinc salts, citric and sulphate salts are also found in oral products	
Polymeric delivery system	1. PLGA	Emulsion evaporation or emulsion diffusion	10–1000	Applications include drug delivery and diagnosis in cardiovascular disease, cancer, vaccine and tissue engineering	
	2. Dendrimers	Divergent growth method	1–10	Applications include transdermal, oral, ocular and pulmonary as well as targeted and/or controlled release	
	3. Cyclodextrins	Coprecipitation method	80–125	Mainly works as solubilizers for compounds with weak water solubility	[65]

NP: Nanoparticle; SLN: Solid liquid nanoparticle.

chemical bodies including peptides and proteins [72]. SLNs are slightly toxic and biocompatible. They lack any organic solvent residues. SLNs are used to target the biofilm-developing microbes rather than developed biofilms.

Microemulsions

Microemulsions are essentially two non-miscible fluids that are composed of water, oil and a surfactant or co-surfactant component that is transparent and thermodynamically stable [73]. Microemulsions are classified into mainly three types, primarily based on the structure assumed by the surfactant component. The three chief categories are oil-in-water (o/w), in which the volume of the oil is lower; one in which the volume of water is less than that of oil, or a water-in-oil (w/o) mixture; and a bicontinuous structure that varies from an o/w to a w/o microsystem. The o/w and w/o microemulsions are made of distinct droplets encircled by a stabilizing layer of surfactant and scattered in an uninterrupted stage [74]. A microemulsion loaded with curcumin was used against planktonic *Streptococcus mutans*, *Lactobacillus casei*, *Actinomyces viscosus* and *Candida albicans*. The findings showed that the microemulsion formulation was superior in yielding more promising results against microorganisms compared with other methods [75]. Microemulsion and nanoemulsion reportedly possess the ability to inhibit the growth of *S. aureus* NCTC 1803, *Streptococcus typhimurium* PSB 367, *Listeria monocytogenes*, *P. aeruginosa* and *E. coli* O157:H7 biofilms [75,76]. Microemulsion has also displayed a greater antimicrobial activity against *P. aeruginosa*. The use of microemulsions is not limited to acting as a drug carrier; some studies reveal that the microemulsions include components that can be used directly against microbial biofilms and show antimicrobial properties. Thermodynamic consistency gives microemulsions the capability to lower the toxic levels of enclosed material. They may deliver hydrophilic, hydrophobic and amphiphilic drugs and have demonstrated controlled release in antimicrobial delivery used for the transfer, topical, ocular and parenteral administration [77,78]. Although the advantages are great, they may also have a few drawbacks, which mainly include the presence of cosolvents like ethanol and surfactants like

polysorbates, which have led to increased toxicity [79]. These events are reported more often in cases where repeated dosages of drugs is needed.

Polymeric-delivery systems

PLGA

PLGA NPs are widely used in medical applications and the pharmaceutical industry due to their wide range of applications, such as slow rate of drug release, biomaterials and scaffolds for tissue engineering and medical devices [80,81]. For instance, microspheres are used for a contained and discharged drug-delivery system due to the wide range of their manufacturing of polymers such as poly(lactic acid), poly(glycolic acid) (PLGA), poly(lactic-co-glycolic acid) and poly(caprolactone) [80].

NPs are usually loaded with active drugs to accomplish a therapeutic activity. In a review, it was shown that several functional groups related to the polymers exhibited antimicrobial activity [82]. Polymeric formulations have been broadly used for the transfer of antimicrobial drugs to the biofilm and biofilm-developing microbes.

Cheow and Hadinoto (2012) discussed the development of rhamnolipid-triggered release from lipid-polymer hybrid NPs, and these rhamnolipids were expressed in *P. aeruginosa* biofilms and other pulmonary-related biofilms [83]. NPs were prepared with a PLGA core surrounded by a lipid coat (PC). The nanoparticles were then enclosed in the fluoroquinolone antibiotics from categories I and IV of the Biopharmaceutical Classification System (BCS). The solubility and the capacity to permeate through intestinal membrane of drugs are classified according to the BCS system [84]. The BCS system classifies the drugs based on the following properties:

1. Category I drugs include levofloxacin and ofloxacin; characteristics: high solvability and high permeability;
2. Category IV drugs include ciprofloxacin; characteristics: low solvability and low permeability.

The category I drugs are freely released from the NPs with or without triggering the agents (i.e., rhamnolipid or Triton X-100). This has demonstrated that NPs are not affected by the lipid coat. To encapsulate category IV drugs into the lipid-polymer NPs, two contrasting PLGA coats were studied: PC/stearylamine (SA) or the polyvinyl alcohol. Only 5% of the drug was deposited inside the polymeric core. The discharge of ciprofloxacin from PLGA-PC/SA was 15% (w/w) after 24 h with or without the triggering agents. The PVA NPs showed an increase in the release to ~25% (w/w) in the absence of triggering agents. As observed in the study, the findings clearly state that the lipid-based coat PC/SA does not directly involve in inhibiting the release of category IV drugs – namely, ciprofloxacin from the core of PLGA NPs. This may be due to the reduced initial drug encapsulation and related lower concentration gradient. A category I drug, levofloxacin, was found to be effective against *P. aeruginosa* biofilm and planktonic cells, showing significant antibacterial activity [85].

PLGA NPs could also be altered to furnish a pH-controlled discharge of antibiotics or drugs like gentamicin sulfate when secured with titanium [86,87]. To achieve a pH-controlled delivery form of NPs, the following procedure was adopted. Ring-opening copolymerization of norbornene was performed with α - ω -functionalized poly(ethylene oxide) macromers. The α -functionalization of the macromonomer was then achieved with the norbornene groups. ω -functionalization with the antibiotic, gentamicin sulfate and carboxylic acid group allows the grafting of the particles to the titanium surface.

Dendrimers

Dendrimers are hyperbranched molecules discovered by Fritz Vogtle in 1978. He named them 'dendron,' which comes from a Greek word, meaning 'tree.' As the name suggests, dendrons are monodispersed macromolecules that possess extremely forked 3D structures. They are capable of delivering not only hydrophobic drugs but also hydrophilic drugs [88]. Dendrimers in general possess a ball-shaped structure containing a polymeric terminus (exo-receptor). These NPs can demonstrate peculiar functionality through the confounded chemistry, differing in their interior endoreceptor binding groups [89,90].

Fucose-specific lectins (Lec-B) are produced by *P. aeruginosa* during the formation of biofilm. Lec-B is integral in the process of biofilm formation, and the mutants that have Lec-B deficiency show dysfunctional biofilm formation that was further examined by Johansson *et al.*, who studied Lec-B inhibition, specifically whether the Lec-B inhibition with elevated affinity polyvalent fucosyl-peptide dendrimers interrupts the binding process and stops the development of biofilm [91]. Johansson *et al.* used a combination library screening approach, allowing them to find one particular ligand, FD2 (C-Fuc-LysProLeu)₄ (LysPheLysIle)₂ (LysHisIleNH)₂, which was capable

of completely suppressing *P. aeruginosa* biofilm formation and dispersing the already developed biofilm formed by *P. aeruginosa* clinical isolates. The study reveals the captivating potential of dendrimers that show antimicrobial and antibiofilm formation activities even in the absence of incorporated antibiotics or antimicrobials attached to the surface of NPs.

Cyclodextrins

Cyclodextrins (CDs) have a hydrophobic interior due to the carbon skeletal structure of glucopyranose monomers and a hydrophilic surface in a truncated cone-shaped structure. CDs are cyclic organic compounds that mainly belong to glucopyranose units coupled with α -1,4-glycosidic linkage [92]. Despite the weak solubility of CD in water, it has several applications because of its unique function as a solubilizer for compounds with weak water solvability. In a recent study, it was found that CDs combined with nitric oxide (NO) were able to eradicate all biofilms by using the same concentration of NO as given in the inhaled formulation [93]. The ability of NO-releasing cyclodextrins against *P. aeruginosa*, which is the main contributor to morbidity and mortality in CF patients, suggests that NO has the potential to become a superior antibiofilm treatment when combined with controlled-release NPs [93].

Chitosan nanoparticles

Chitosan, a polysaccharide molecule derived from chitin is one of the most abundant polymers found in nature. The substance is made by (β)-1,4 d-glucosamine linked to N-acetyl-d-glucosamine residues [94]. It displays unique properties, including biodegradability and biocompatibility, which make it a good choice for researchers. It is also a cost-effective alternative for many uses in food safety and biomedicine [95]. Chitosan also possesses antibacterial and anti-biofilm properties against *S. aureus*, *E. coli*, and *S. typhimurium* [96]. Chitosan NPs are formulated through ion gelation with TPP (polyanionic sodium triphosphate) [97].

The efficiency of chitosan nanoparticles against *S. mutans* was reported [98]. Chitosan and its interactions with the bacterial biofilm have been explained in several ways [99,100]. One of the proposed mechanisms describes interactions between the positively charged chitosan molecules and the negatively charged microbial cell membranes, leading to the leakage of proteinaceous and some other intracellular constituents [101,102]. Chitosan NPs can retain their charge even at neutral pH. The other mechanism proposed is the binding of chitosan NPs with microbial DNA, which interferes with the mRNA and protein synthesis in the bacterial cells of the biofilm after the bacteria enters the cell.

The study of chitosan NPs against *S. mutans* biofilm revealed the antimicrobial activity of chitosan NPs, which are efficient at pH 7. Chitosan NPs with a lower molecular weight show a stronger trend toward higher particle activity. In addition, the chitosan NPs with a lower molecular weight may affect the cohesion of the cell membrane of *S. mutans* in a homogenous fashion throughout the entire biofilm. Given the successful results of this study, it may be concluded that the system could be improved even further with uniform delivery of chitosan NPs at a neutral pH throughout the biofilms. It is modified by combining it with other compounds. Chitosan NPs may aid in targeting highly potent and variable microorganisms in a complex biofilm system if modified with suitable compounds [98].

Inorganic NPs

Silver NP formulations

Silver NPs (AgNPs) are acclaimed for their prominent antimicrobial activity. Silver NPs are used against the biofilms of *S. epidermidis* and *P. aeruginosa* [103].

Silver NPs can be prepared by several methods [104]. AgNPs are reported to successfully inhibit the growth and biofilm formation of both Gram-negative and Gram-positive bacterial strains, even at a very low concentration, compared with the minimum inhibitory concentration breakpoints of other antibiotics. Furthermore, AgNPs displayed no reduction or cytotoxicity against mammalian cells [105].

Another study also reported that *P. aeruginosa* PAO1 biofilms were inhibited by treatment with silver NPs [106]. The purpose of this study was first to determine the potency and effectiveness of the citrate-capped AgNPs of various dimensions alone and, second, AgNPs amalgam with monobactam antibiotics, aztreonam to inhibit the targeted biofilm. The findings suggested that potentially smaller dimension AgNPs enhanced the antimicrobial effects of aztreonam against *P. aeruginosa in vitro*, revealing a potential role for amalgams of smaller size AgNPs with antibiotics in the treatments of patients with chronic pulmonary infections and diseases. Therefore, the role of silver NPs in chronic respiratory infection treatment has much potential for further research.

Gold NPs

A recently reported study discussed the use of phosphatidylcholine-coated gold NPs packed with gentamicin (GPA-NPs) [107]. The GPA-NPs had a size of 180 nm, and a uniform size of NPs was used. The findings revealed that the GPA-NPs sustain their antibiotic mechanisms against planktonic bacteria, compelling to impair the settled biofilms. The results revealed the successful inhibition of biofilm formation of both Gram-negative and Gram-positive pathogenic bacteria. Moreover, the GPA-NPs also displayed a nontoxic trait in RAW264.7 cells. Therefore, it may be concluded that GPA-NPs might play a crucial role as a potential antimicrobial agent with promising and effective treatment of chronic pulmonary infections due to intracellular bacteria and microbial biofilms.

Recent research has cast light on the biofilm-inhibiting property and bactericidal activity of such NPs on *S. aureus* and *P. aeruginosa* [108]. By reducing the surface charge of AuNPs, cell toxicity was decreased. The *in vitro* studies on AuNPs propose that the modulations of the surface charge of AuNP may be exercised to stabilize the effects on bacteria against airway cells in ventilator-associated pneumonia (VAP).

Silica NPs

Some pathogenic strains of bacteria have the potential to withstand antibiotics. This has led to the search for novel approaches against microbial resistance and potential therapies against the development of infectious biofilms in patients with pulmonary infections. Silica NPs (SiNPs) are a good option to deliver drugs, such as antibiotics against biofilms and to show therapeutic inhibition and antimicrobial effects in chronic infections.

Pinto *et al.* reported on NO-encapsulated SiNPs to target biofilms of *S. aureus* and *P. aeruginosa* [109]. In this study, NO showed antimicrobial activity and cell death. With an increased aspect ratio and reduced nanoparticle size, NO-SiNPs were considerably effective against both biofilms of *S. aureus* and *P. aeruginosa*, whereas the Gram-negative species exhibited higher sensitivity to NO. The conclusions also stated that the smaller the size of the NP formulations, the greater the efficacy of the NO antimicrobial properties and better delivery. The shape of the NP was another factor in improving the function of NO. The rod shape of SiNPs showed a better delivery than normal circular SiNPs.

Conclusion

It is estimated that approximately 60% of chronic or recurrent infections have a direct link to biofilms. Therefore, to treat biofilm-associated respiratory infections, several extensive studies have been ongoing across the globe. Several potent drug molecules have been studied and suggested to inhibit multiresistant biofilms and to suppress their actions. Two of the effective strategies, inhaled formulations and nanoparticles, were discussed in detail in this review.

A significant feature of NP delivery is the size range of the particles administered, which are $<1 \mu\text{m}$ in size, compared with many other drug-delivery techniques which have particles $<5 \mu\text{m}$ in size. Furthermore, NPs uniformly distribute the targeted drug in alveolar regions, which could improve drug targeting in the lungs. This has also been shown to increase the surface area of the drug per unit mass and maximize the accumulation of the drug in the lungs.

Apart from such drug-delivery techniques, another effective way of managing respiratory infections is by providing inhaled formulations as treatment modalities. Inhaled formulations may target biofilms that cause respiratory infections. In addition to the parenteral and oral delivery techniques, inhaled formulations may be used in combination, which could prove promising in the near future. The use of NPs holds huge potential, and NO-containing NP formulations were found to be the most promising and suitable for therapeutic use.

Future perspective

Biofilm infections are common and clinically significant, resulting in potentially fatal infections. However, our understanding of their role in chronic pulmonary infections is still limited. The emerging evidence also implies that biofilms are not just limited to bacteria but also extend to other infections such as those produced by mycobacteria and *H. influenzae*. The treatment of biofilm infections is hindered by several potential challenges, as discussed in detail in this review. These factors limit the applications of current antibacterial and antifungal drugs. Nevertheless, the application of nanotechnology seems to provide some interesting evidence as a potential treatment modality in the treatment of various biofilm-associated infections in chronic respiratory diseases. This novel approach holds promising *in vitro* and *in vivo* potential. However, there is still an urgent need for in-depth mechanistic and translation-oriented studies before such advanced strategies can be used in regular clinical practice.

Executive summary

Biofilms: Multiresistant properties & prevention

Multidrug resistant properties in biofilms

- Exopolysaccharides provide delay in the penetration of antimicrobial drugs and are one of many contributing factors to multidrug resistance.

Heterogeneity

- The bacterial cell culture shows a period of slow or no growth during limited supply of nutrition, and this transition is suggested to be a critical factor leading to antibiotic resistance.

Prevention of biofilms

Various types of biofilms in respiratory infection

- *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, *Mycobacterium abscessus*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Staphylococcus* spp. are predominantly seen in respiratory infections that form biofilms.

Diagnosis of biofilms

- Biofilm infections are complex to diagnose by conventional methods of sampling or through microbiology tests.
- Biofilm infections could also relapse or fail to respond to antimicrobial therapies.

Challenges in treatment strategies

- The current methods of treatment for biofilm infections are not efficient and face many complicated hurdles for microbiologists and clinicians.
- Multidrug resistance and varied metabolic activity pose as a challenge in biofilm treatment.

Current methods of treatment

Antibiotic treatment (high concentrations) administered through topical route

- Antibiotic treatment, if used as a sole method to treat biofilm infection, might not yield the best results.

Combined & sequential antimicrobial therapies

- Biofilm infection treatment will require the collaboration of other therapies, including clinical microbiology, surgery, internal medicine and basic and clinical pharmacology.
- Understanding the nature of microbial biofilms will help in determining the combined and sequential therapies against it.

Inhaled antimicrobial formulations: treatment of biofilm-induced respiratory infections

- Inhaled formulations have relatively shown positive outcomes *in vitro*. However, they were not successful in their ability to reach the site of action *in vivo*.

Quorum sensing (QS) inhibitors & anti-QS peptides to control biofilm infection

- It has been suggested that the current biofilm treatments should use strategies to penetrate biofilms. In addition, it is suggested that sensitive antibiotics combined with high-dose antibiotics be employed along with QS treatment to successfully disperse and kill microbes in a biofilm.

Antagonists of diguanylate cyclase (DGC)

- The use of inhibiting compounds of DGC showed damage to *P. aeruginosa* and *Acinetobacter baumannii* biofilms.

Bacteriophage therapies

- Bacteriophage therapies were able to show positive results in implant-, biofilm-related infections and wound-related biofilm infections due to their ability to infect and kill both of antibiotics sensitive and resistant bacteria.

Nanoparticle Drug-Delivery Formulations

Lipidic-delivery systems

Liposomes

- The liposome-based nanoparticles (NPs) when loaded with linezolid against methicillin-resistant *Staphylococcus aureus* (MRSA) have showed potential antibiofilm activity. The liposomal NPs might be efficient against osteomyelitis causing MRSA, which is difficult to treat.

Solid lipid nanoparticles

- The solid lipid NPs are utilized specifically to target the developing biofilms rather than developed biofilms.

Microemulsions

- Microemulsions loaded with curcumin was successfully employed against planktonic *Staphylococcus mutans*, *Lactobacillus casei*, *Actinomyces viscosus* and *Candida albicans*. The findings revealed that microemulsion-loaded formulation was better in yielding more promising results against microorganisms.
- Microemulsions have the potential to be used as therapeutic agent alone or as a drug carrier.

Polymeric-delivery system

Dendrimers

- Lec-B, an essential agent required for biofilm growth, is inhibited by polyvalent fucosyl-peptide dendrimers.
- Studies have revealed the captivating potential of dendrimers that show antimicrobial and antibiofilm formation activity, even in the absence of incorporated antibiotics or antimicrobials attached to the surface of NPs.

Cyclodextrins

- Nitric oxide (NO)-releasing cyclodextrins have shown promising results in recent studies in completely eradicating the *P. aeruginosa* biofilm in patients.
- Cyclodextrins may become efficient candidates for research against antibacterial biofilm in respiratory diseases.

Inorganic delivery system**Gold NPs**

- Gold NPs have shown potent antimicrobial activity against *S. aureus* and *P. aeruginosa*.
- Toxicity could be reduced in Gold NPs by reducing the surface charge.

Silver NPs

- Silver NPs have shown successful antimicrobial activity against *S. epidermidis* and *P. aeruginosa* biofilms.
- They can be used at a very low concentration and do not result in cytotoxicity in mammalian cells.

Silica NPs

- NO-silica NPs (siNPs) were considerably productive against both biofilms of *S. aureus* and *P. aeruginosa*, whereas the Gram-negative species exhibited higher sensitivity to NO.
- The rod-shaped SiNPs showed better delivery than normal circular-shaped SiNPs.

Chitosan NPs

- Chitosan NPs, when used with phosphatidylcholine and gentamicin, were able to improve the penetration of drugs into the biofilm and engulfment of NPs by macrophages with a negligible cytotoxicity to cells.

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