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Combination and nanotechnology based pharmaceutical strategies for combating respiratory bacterial biofilm infections

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ABSTRACT

Respiratory infections are one of the major global health problems. Among them, chronic respiratory infections caused by biofilm formation are difficult to treat because of both drug tolerance and poor drug penetration into the complex biofilm structure. A major part of the current research on combating respiratory biofilm infections have been focused on destroying the matrix of extracellular polymeric substance and eDNA of the biofilm or promoting the penetration of antibiotics through the extracellular polymeric substance *via* delivery technologies in order to kill the bacteria inside. There are also experimental data showing that certain inhaled antibiotics with simple formulations can effectively penetrate EPS to kill surficially located bacteria and centrally located dormant bacteria or persisters. This article aims to review recent advances in the pharmaceutical strategies for combating respiratory biofilm infections with a focus on nanotechnology-based drug delivery approaches. The formation and characteristics of bacterial biofilm infections in the airway mucus are presented, which is followed by a brief review on the current clinical approaches to treat respiratory biofilm infections by surgical removal and antimicrobial therapy, and also the emerging clinical treatment approaches. The current combination of antibiotics and non-antibiotic adjuvants to combat respiratory biofilm infections are also discussed.

1. Introduction

Respiratory infection is one of the leading global health threats. Among these, chronic respiratory infections caused by biofilm-growing bacteria in cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and bronchiectasis patients are generally difficult to treat and eradicate (Høiby et al., 2011; Weers, 2014). The formation of biofilms is the strategy used by microorganisms to survive in nature and disease

Abbreviations: ASL, Airway surface liquid; Au, Gold; BALT, Bronchi-associated lymphoid tissue; CF, Cystic fibrosis; COPD, Chronic obstructive pulmonary disease; CR, Chronic rhinosinusitis; Cu, Copper; c-di-GMP, Bis-(3′-5′)-cyclic dimeric guanosine monophosphate; Chol, Cholesterol; DNase, Dornase alfa; DPn, Degree of polymerization; DPPC, Dipalmitoylphosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DDAB, Didecyldimethylammonium bromide; DAP, 1,2-dioleoyl-3-dimethylammonium-propane; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DP, dry powder; EPS, Extracellular polymeric substance; eDNA, Extracellular DNA; EDTA, Ethylene diamine tetra acetic acid; Fe (III), Ferric iron; Fe(II), Ferrous iron; FDA, US food and drug administration; Fe_3O_4, Ferroferric oxide; IL, Interleukin; INF-γ, Interferon-gamma; IU, International units; LF, Liquid formulation; LI, Lung infection; MBEC, Minimum Biofilm Eradication Concentration; MIC, Minimum Bactericidal Concentration; Mw, Molecular-weight; MgF_2, Magnesium halogen; NO, Nitric oxide; NF-κ β, Nuclear Transcription Factor-Kappa β; NO_3^-, Nitrate; NO_2^-, Nitrite; N_2O, Nitrous oxide; NAR, Nitrate reductase; NIR, Nitrite reductase; NOR, Nitric oxide reductase; N_2OR, Nitrous oxide reductase; N/A, Not available; NCFB, Non-cystic fibrosis bronchiectasis; NLCs, Nanostructured lipid carriers; NPs, Nanoparticles; O_2, Oxygen; O^{2-}, Superoxide; PMNs, Polymorphonuclear leukocytes; PCD, Primary ciliary dyskinesia; PK, Pharmacokinetic; PD, Pharmacodynamic; PC, Phosphatidylcholine; PL, Poly (L-lysine); PLGA, Poly (lactic-co-glycolic) acid; PCL, Poly caprolactone; PVA, Polyvinyl alcohol; PEG, Polyethylene glycol; PDEs, Phosphodiesterases; QSIs, Quorum sensing inhibitors; QS, Quorum sensing; ROS, Reactive oxygen species; RNOS, Reactive nitrogen oxide intermediates; sIgA, Secretory Immunoglobulin A; SLNs, Solid lipid nanoparticles; TOBI®, Tobramycin; TiO_2, Titanium dioxide; ZnO, Zinc oxide.

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(Costerton et al., 1987; Costerton et al., 1999). Biofilms are structured communities of bacterial aggregates that are adherent to inert or living surfaces or situated in the tissue or mucus. What makes these structures special is that living within these biofilm communities makes its resident bacteria tolerant to antibiotics (Hoiby et al., 2015). Some microorganism *e.g., Pseudomonas aeruginosa* or *Streptococcus mutants* transforms into mucoid phenotypes in mucus during chronic lung infections or on dental surfaces by production of extracellular polysaccharides, which makes them physiologically able to resist the innate and adaptive inflammatory defense mechanisms and antibiotic treatments (Powell et al., 2018). Different from biofilms that attached to surfaces, the biofilms of chronic respiratory infections are typically embedded into highly viscous mucus, but not directly at the epithelial cell surfaces (Hassett et al., 2002), providing a significant physical barrier to the antibiotics, biocides as well as phagocytic cells (Hassett et al., 2010). The matrix of the biofilm growing bacteria can actually be regarded as an independent pharmacological microcompartment (Cao et al., 2016; Cao et al., 2015; Christophersen et al., 2020).

There is a gradient of oxygen from the surface to the center of biofilms and therefore of bacterial activities due to consumption of oxygen (O_2) by the activated polymorphonuclear leukocytes (PMNs) around the biofilms, resulting in anaerobic conditions in the center of the biofilm and therefore dormant bacterial cells (Smith, 2005). Biofilm growth lead to a significant decrease in susceptibility to antimicrobial agents compared with planktonic bacteria (Smith, 2005), but re-oxygenation by hyperbaric oxygen treatment restores the activity of some antibiotics (Kolpen et al., 2016). Consequently, the Minimum Biofilm Eradication Concentration (MBEC) of some antibiotics for treating bacteria in the biofilms are 100–1000 times higher than the Minimum Bactericidal Concentration (MIC) for treating the same planktonically growing bacteria (Ceri et al., 1999; Roy et al., 2018). Moreover, the bacteria in the biofilms can additionally be resistant due to traditional mechanisms (*e.g.,* beta-lactamase production) to a variety of antibiotics (Bagge et al., 2004).

The current clinical approach to combat the respiratory biofilm infections still primarily rely on antibiotic management, although surgical removal is also used in some cases (Klodzinska et al., 2016). Surgical removal of the biofilms is not feasible in CF though, except in the paranasal sinuses or patients with lung transplantation (Wilkins et al., 2014). The antibiotics are mainly administered *via* oral and/or intravenous routes depending on the disease severity (Hoiby, 2011), which can maintain the lung function for decades (Fernandez-Barat et al., 2017). Inhaled antimicrobial agents has also been used for several decades in CF (Heijerman et al., 2009), as it can directly deliver the antimicrobial agents into the airways resulting in high local concentration, and reduced side effects (Klodzinska et al., 2016). However, the administration of the antibiotics *via* inhalation or systemic administration routes does not clear the chronic respiratory biofilm infections and in many cases, the biofilms become mutationally resistant to the antibiotics (Ciofu et al., 2015; Fernandez-Barat et al., 2017). Several mechanisms for drug resistance and drug tolerance of the biofilms have been reported such as limited diffusion of the antimicrobial agents to the increased mucus and biofilm matrix, the covalent inactivation of antimicrobial agents by the enzymes in extracellular polymeric substances (EPS) matrix or other matrix components, and an altered bacterial physiological activity in the biofilms including dormant cells as discussed above (Borriello et al., 2004; Koo et al., 2017). To overcome these mechanisms of drug resistance and tolerance, some new treatment strategies have been proposed.

One of the promising approaches to combat the drug resistance of the biofilm infections is to use adjuvants in the combination with antibiotics (Brooks and Brooks, 2014). The co-administration of multiple bio-active agents has shown to be effective against respiratory biofilms, which could confer adequate therapeutic effects at reduced doses (Brooks and Brooks, 2014). The adjuvants used in the combination therapy could target more than one component of the complex biofilm microenvironment, which could eventually overcome the drug resistance (Li et al., 2006). In this context, the lipid and polymer-based nanotechnology-based drug delivery systems are commonly exploited, which are biocompatible, biodegradable, and relatively stable as well as being practical to produce (Ho et al., 2019). In addition, several other types of nanotechnology-based drug delivery systems, including metal-containing nanoparticles, nitric oxide (NO)-releasing nanoparticles, stimuli-triggered nanoparticles are also used to combat biofilm-evoked drug resistance. These nanoparticles commonly facilitate the delivery of the antimicrobial agents to the sites of infection at their highest doses, thereby overcoming the drug resistance with reduced adverse effects to the patients (Leid et al., 2012).

This article aims to review the recent advances in pharmaceutical strategies for combating biofilm growing bacteria that are embedded in the airway mucus layer in the respiratory tract with a focus on nanotechnology-based drug delivery systems. Recent advances in combining antibiotics with non-antibiotic adjuvants to treat chronic respiratory infections caused by biofilm and the challenges and future opportunities of inhaled medicines combating respiratory biofilm infections are also discussed.

2. Respiratory biofilm infections

The respiratory biofilms are unique for pathogens, where the bacteria located in the conductive zone of the lungs are embedded in the airway mucus layer, functioning as a breed ground for bacteria (Hoiby, 1974). Pulmonary diseases such as CF, COPD, primary ciliary dyskinesia (PCD) (Alanin et al., 2015) and bronchiectasis can lead to high mucus secretion, which is accompanied by impaired mucociliary clearance (Weers, 2014). When the biofilms reach the respiratory zone of the lungs, inflammation is initiated and acute exacerbations can occur and the lung function is worsened (Bjarnsholt et al., 2010). The progression of these diseases further leads to chronic infection of various bacteria, resulting in clinical manifestations of biofilm infections, including chronic endobronchial bacterial infections and airway inflammation. This also causes airway obstruction, progressive destruction of the airway epithelium, ultimately resulting in respiratory failure (Marras et al., 2018; Park et al., 2016). A model of *P. aeruginos*a biofilms in hypoxic CF mucus is illustrated in Fig. 1, and some characteristics of respiratory biofilm infections are presented below.

2.1. Biofilms embedded in the mucus

The mucus is the first landing port of entry in the lungs for invasive bacteria, providing proper environment for the pathogenic infections and reducing the susceptibility to various antibiotics on bacteria (Merchant et al., 2016). Using electron microscopy and lung tissue explants, studies show that CF patients with chronic lung disease directly, had *P. aeruginosa* present in mucopurulent hypoxic microcolonies of approximately 100 μm diameter in the airway lumen, rather than attached to the epithelium (Worlitzsch et al., 2002). Normally, the airway epithelial cells have a thin, hydrated mucus layer located on the top of airway surface liquid (ASL) (Munder and Tuemmler, 2015). At normal physiological condition, the viscosity of ASL is low, which do not hinder the mucociliary clearance mechanism (Powell et al., 2018). However, in pulmonary disease conditions, *e.g.,* CF, a dehydration in the ASL leads to an increase in the viscosity of ASL and accumulation of mucus in the lower airways. This impairs the mucociliary clearance and facilitates colonization and infection of the lower airway environment by bacterial pathogens, especially *P. aeruginosa*, the major morbidity and mortality cause in the CF population (Munder and Tuemmler, 2015). This can further develop into a chronic stage of infection, marked by a biofilm-growing *P. aeruginosa* leading to the main clinical feature of CF (Koehler et al., 2004). The increased viscosity of CF airway mucus acts as a matrix scaffold (Torge et al., 2017), in addition to the biofilm matrix (Cao et al., 2016), obstructs the diffusion of antibiotics to reach biofilm.

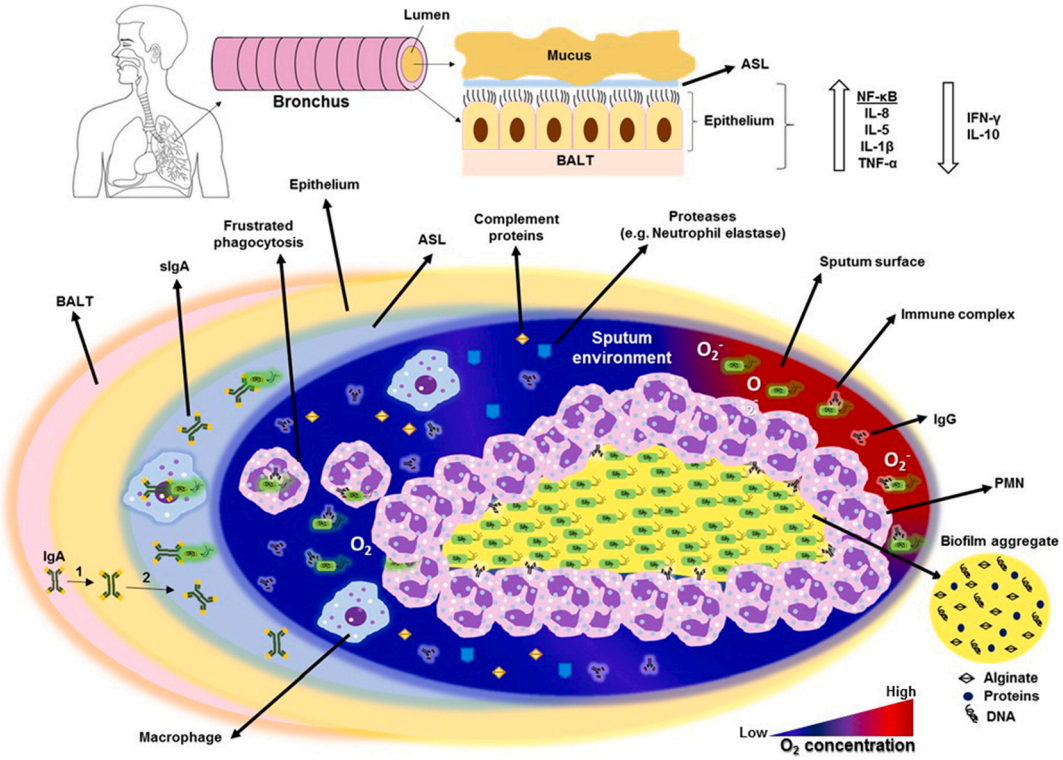

Fig. 1. Model of *P. aeruginosa* biofilms in hypoxic CF mucus. Mucus with a *P. aeruginosa* biofilm surrounded by PMNs and IgG antibodies specific to *P. aeruginosa* antigens, such as alginate, lipopolysaccharide and proteins. Immune complexes are formed between *P. aeruginosa* antigens and IgG, which activate complement and attract PMNs. Activated PMNs consume oxygen and liberate reactive oxygen species (ROS), proteases and DNA, starting the inflammatory reaction. Such inflammatory reaction consumes all oxygen in mucus, which becomes anaerobic, impairing the PMNs' ability to phagocytose and kill *P. aeruginosa* in sputum due to the oxygen starvation needed for PMN activity, and leading to a frustrated phagocytosis. The pronounced IgG antibody response occurs especially due to the high Nuclear Transcription Factor-Kappa β (NF-κ β) production by CF airway epithelial cells, and to the T-cell (Th2)-skewing of the adaptive immune response, resulting in overproduction of proinflammatory cytokines interleukin (IL), like IL-5 and IL-8, and low production of anti-inflammatory cytokines, like IL-10 and interferon-gamma (INF-γ). Since the PMNs' ability is impaired, this hyperinflammatory response leads to tissue damage rather than killing *P. aeruginosa* biofilms. IgA is produced in the bronchi-associated lymphoid tissue (BALT) submucosa (1), where it combines with the secretory component, yielding secretory Immunoglobulin A (sIgA), which is exported through the epithelial cells to the airway surface liquid (ASL) (2), preventing *P. aeruginosa* to attach to the epithelial cells, in a macrophage-mediated non-phlogistic response, thereby not contributing to airway inflammation. Adapted with permission from (Mauch et al., 2018).

The most important molecule concerning viscosity of the mucus is extracellular DNA (eDNA) released by disintegrating inflammatory cells, particularly PMNs, which is abundant in infected CF sputum (Hodson, 1995). This eDNA also binds to positively charged aminoglycoside antibiotic (Chiang et al., 2013), such as tobramycin, protecting the bacteria from tobramycin (Tseng et al., 2013). That is the background for CF patients being treated with dornase alfa (DNase) inhalation for more than two decades (Hodson, 1995).

Various studies have demonstrated that the mucus is not a homogenous fluid, and the mucus layer shows highly non-uniform aperture distribution with pores smaller than the conventional drug particles (Duncan et al., 2016). Moreover, the polymeric mucins and other biomacromolecules could cross-link through disulfide bonds and/or physically entangle to form a mesh-like structure, hindering drug penetration (Duncan et al., 2016). The last but not the least, most glycoproteins present in the mucus layer have higher sialic acid and sulfate contents, which could create negatively charged surfaces, showing a greater affinity to positively charged antibiotics and might further reduce the activities of these antibiotics against *P. aeruginosa* (Costerton et al., 1999).

2.2. Emergence of the mucoid phenotype

In the early stages of disease, the non-mucoid phenotype of *P. aeruginosa* is present as planktonic organisms in sputum in the conducting airways, which is frequently succeeded by colonization-treatment-recolonization cycles (Folkesson et al., 2012). This can further develop into a chronic stage of infection, marked by a mucoid phenotype (biofilm mode of growth) (Table 1). A similar biofilm strategy is also adopted by *Burkholderia, Achromobacter and Stenotrophomonas species* and *Mycobacterium absessus* (Qvist et al., 2015). The mucoid *P. aeruginosa* phenotype results from bacterial hyperproduction of a polysaccharide known as alginate or mucoid exopolysaccharide

Table 1
The properties of mucoid and non-mucoid phenotypes of *P. aeruginosa* in the respiratory tract of patients with CF. Modified with permission from (Ciofu et al., 2015).

Property	Non-mucoid phenotype	Mucoid phenotype
Stage of emergence	Early stage	Late stage
Position in the lungs	Conducting airways in sputum	Respiratory zone in alveoli and conducting airways in sputum
Biofilm formation *in vitro*	Yes	Yes
Biofilm formation *in vivo*	No	Yes
Resistance due to biofilm properties	No	Yes
Multiply antibiotic resistance due to conventional mechanisms	Frequent	Seldom
Antibody response	No	Yes (Th2 antibody response)
Responsible for lung tissue damage	No	Yes

rendering the bacteria resistant to phagocytosis of both PMNs and macrophages (Bjarnsholt et al., 2010; Ciofu et al., 2008). Although all of the potential mechanisms of conversion to mucoid in CF are not yet defined, a major one is thought to be through mutations in *mucA* (Mathee et al., 1999), encoding a member of the extra cytoplasmic function family of anti-sigma factors (Martin et al., 1993). However, clinical mucoid isolates that carry no mutation within the *mucABCD* cluster, have been reported (Hassett et al., 2010). Recently, it was shown that the induction of an envelope protein called *mucE* (Qiu et al., 2007) and mutations in the sensor kinase *kinB* (Damron et al., 2009) results in alginate overproduction in *P. aeruginosa* strains with the wild-type *mucA*. Therefore, there are at least two pathways that govern the conversion to mucoid in *P. aeruginosa* (Hassett et al., 2010). In short, the mucoid phenotype severely complicates the overall clinical prognosis for patients with CF and COPD and enhances their antibiotic- and phagocyte-resistant properties (Anwar et al., 1989).

2.3. Anaerobic metabolism of P. Aeruginosa

In the chronic lung infection, *P. aeruginosa* biofilm are surrounded by numerous PMNs in the endobronchial mucus, where the activity of PMNs is the major cause of O_2 depletion rendering the *P. aeruginosa* biofilm anoxic (Jensen et al., 2017; Mauch et al., 2018). The absence of O_2 in parts of the CF airways has been further confirmed by the isolation of obligate anaerobes from CF sputum and bronchoalveolar lavage fluids (Su and Hassett, 2012). PMNs accumulating around *P. aeruginosa* biofilm aggregates can induce intense O_2 consumption and the production of ROS and the formation of NO, thereby impeding aerobic respiration of *P. aeruginosa* (Jensen et al., 2017). However, *P. aeruginosa* can adapt to such PMN-induced microenvironmental changes *via* anaerobic respiration in the anoxic zones of endobronchial mucus, which is significant different from those of test-grown cells, and even from those of aerobic biofilms (Cowley et al., 2015). The process of anaerobic metabolism by denitrification involving utilization of the alternative electron acceptors, nitrate (NO_3^-), nitrite (NO_2^-) or nitrous oxide (N_2O), that are present in CF airway mucus (Hassett et al., 2002; Jensen et al., 2017). Complete bacterial denitrification is performed by the four enzymes, *i.e.,* nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR), which catalyze the four-step reduction of NO_3^- to N_2 (Jensen et al., 2017):

$$NO_3^- \text{ (NAR)} \rightarrow NO_2^- \text{ (NIR)} \rightarrow NO \text{ (NOR)} \rightarrow N_2O \text{ } (N_2OR) \rightarrow N_2 \quad (1)$$

Analyses of anaerobe pathogenicity may also yield insight into the relationships between anaerobes and airway disease severity (Muhlebach et al., 2018). Many frontline antibiotics used in the treatment of CF airway disease are either ineffective or show reduced killing efficacy in the anaerobic conditions (Hassett et al., 2002). Yet, tolerance toward antibiotics in biofilms is recognized as a major cause of therapeutic failure during chronic infection and the mechanisms of antimicrobial tolerance *in vivo* are not completely understood (Jensen et al., 2017).

3. Current treatment of respiratory biofilm infections

Various approaches to combat the biofilms have been reviewed elsewhere (Klodzinska et al., 2016; Koo et al., 2017), which include the disruption of the biofilm by using physical approaches, antibiotic management, and application of other modalities, such as antimicrobial peptides. It is also possible to modify the surface properties of some medical devices to prevent the bacteria attachment and biofilm formation after implantation. However, for the biofilm infections in the respiratory tract, many physical and chemical approaches become not feasible because the lung is rather fragile, which cannot stand certain mechanical stress and some chemical stimulus. In this section, we briefly review some current treatment approaches to combat respiratory biofilm infections in the clinical setting.

3.1. Surgical removal

Due to the complex physiological structure of the lungs, surgical removal is not the primary approach to treat respiratory biofilm infections. However, the surgical approach is utilized to interfere and remove the established respiratory biofilms in the paranasal sinuses, preventing the spread to the lungs through aspiration. Therefore, an early treatment of the sinus infection by surgical removal approach is clinically recommended (Aanaes et al., 2015). In addition, the physical removal of the mucus infectious through surgical interventions could relieve the breathing obstruction (Rowe-Jones and Mackay, 1996).

3.2. Antimicrobial therapy

In 2014, European Society for Clinical Microbiology and Infectious Diseases published a rather comprehensive guideline for the diagnosis and treatment of biofilm infections (Hoiby et al., 2015). In this guideline, antibiotic management may be the most recommended approach for combating the biofilm infections occurred in the respiratory tract. In CF, antibiotics are used in four different situations: prophylaxis, pre-emptive, empiric or definitive therapy and chronic suppressive therapy (Hoiby et al., 2015). Biofilms can be prevented by early aggressive antibiotic pre-emptive systemic and/or nebulized antibiotic therapy on early *P. aeruginosa* colonization (Hoiby et al., 2015), or can be treated by chronic suppressive therapy. Chronic suppressive antibiotic therapy (maintenance therapy) is given continuously as nebulized antibiotics, supplemented with systemic antibiotic therapy either regularly every 3 months or at acute exacerbations. The patient's lung function can thereby be maintained for decades (Hoiby et al., 2015).

Since both the respiratory and the conductive zones of the lungs are infected with biofilm, it is possible to let antibiotics reach the both compartments of the lungs through the combined modes of antibiotic administration (*i.e.,* topical and systemic) (Hoiby et al., 2015), where inhaled antibiotics mostly deposit at the conductive zones and the antibiotics taken orally or by injection can reach the respiratory zones *via* diffusion from the systemic circulation. Table 2 enlists inhaled antibiotics and other non-antibiotic products, which have been approved or are under clinical trials to treat respiratory biofilm infections. For example, tobramycin (TOBI®) is widely used for the treatment of chronic respiratory infections owing to its proven efficacy against *P. aeruginosa* biofilm (Herrmann et al., 2010).

Although the existing inhaled antibiotic therapy for biofilm infections is usually used to relieve biofilm infections, the delivery of conventional inhaled formulations also inevitably suffers from low antibiotic exposures to the biofilms due to the clearance mechanism of the lung. In the long run, the low antibiotic exposure to the biofilms would give rise to the emergence of antibiotic resistant bacteria. Thus, these challenges, *i.e.,* the low antibiotic exposure in the vicinity of the biofilm colonies due to the short antibiotic retention in the lungs and the presence of the mucus barriers, have to be addressed in the antibiotic therapy with novel therapeutic technologies, *i.e.,* combining antibiotics with other non-antibiotic adjuvants, and nanotechnology-based drug delivery systems.

4. Combination strategy for combating respiratory biofilm infections

Although topical (Priemel et al., 2018; Wang et al., 2020b) and systemic administration of antibiotics are proven to be effective for the treatment of chronic respiratory infections, eradication of biofilms has always been extremely challenging. As one of pharmaceutical strategies, the combination of antibiotics has been increasingly studied to combat respiratory infections (Wang et al., 2020a; Wang et al., 2016). The combination of tobramycin and colistin is a typical example, which exhibited better therapeutic effects than their respective single antibiotics for killing *P. aeruginosa* in biofilms *in vitro*, and they significantly

Table 2
The inhaled antibiotic products and other non-antibiotic products marketed or under clinical trials for the treatment of respiratory biofilm infections.

Drug	Development status	Dosage	Frequency	Condition	Ref.
Tobramycin	Marketed	300 mg	Twice daily	CF	(Ballmann et al., 2011)
Aztreonam	Marketed	75 mg	Three times daily	CF	(Ballmann et al., 2011)
Colistimethate sodium	Marketed	1000000 IU	Twice daily	CF	(Velkov et al., 2015)
Azithromycin	Marketed	N/A	N/A	CF	(Klodzinska et al., 2016)
Levofloxacin	Marketed	240 mg	Twice daily	CF	(Schwarz et al., 2021)
Amikacin	Marketed	590 mg	Once daily	LI	(Zhang and Hill, 2021)
Ciprofloxacin	Phase 3 (NCT02106832) *	32.5 mg	Twice daily	NCFB	(De Soyza et al., 2018)
Tobramycin/Colistin	Phase 3 (NCT03341741) *	112 mg /1 Mega	Twice daily	CF	(Herrmann et al., 2010)
Tobramycin/Fosfomycin	Phase 2 (NCT00794586) *	20 mg/80 mg	Twice daily	CF	(Trapnell et al., 2012)
OligoG	Phase 2 (NCT02157922) *	N/A	N/A	CF	(Weiser et al., 2021)
NO	Phase 1 (NCT01958944) *	160 ppm	Three times daily	CF	(Deppisch et al., 2016)
Silver Colloid	Phase 1/2 (NCT02403479) *	6.7 mcg	Twice daily	CR	(Scott et al., 2017)
Xylitol	Phase 1/2 (NCT00928135) *	7%/15%	Twice daily	CF	(Singh et al., 2020)
Povidone-Iodine	N/A (NCT04278950) *	0.1%	Once daily	CR	(Wu et al., 2021)

N/A, not available; NO, nitric oxide; CF, cystic fibrosis; LI, lung infection; NCFB, non-cystic fibrosis bronchiectasis; CR, chronic rhinosinusitis; IU, international units. * More information on clinicaltrials.gov.

reduced *P. aeruginosa* cell counts in a rat lung infection model and in patients with CF (Herrmann et al., 2010). In addition, this combination could eliminate different antibiotic tolerant subpopulations, and circumvent the occurrence of mutations and antibiotic resistance in biofilm bacteria (Pamp et al., 2008). The antibiotic combinations have been reviewed elsewhere (Sabina, 2015), in this section, we focus on the studies on combining antibiotics with other non-antibiotic adjuvants, including quorum sensing inhibitors (QSIs), EPS interfering agents, metal chelators and bacteriophage to combat the biofilm infections (Fig. 2). The primary objective of these combination therapies is to achieve synergistic therapeutic effects by targeting different bacterial growth pathways, while minimizing the drug resistance.

4.1. Combination of antibiotic and quorum sensing inhibitors

Quorum sensing (QS) is a cell-to-cell signaling cascade coordinating the gene expression of bacteria and it is associated to the population density (Solano et al., 2014). Based on the mechanism of QS signaling (Jack et al., 2018), quorum sensing inhibitors (QSIs) interfering with this regulatory network have been attempted to combat biofilm infection in combination with antibiotics. QSIs differ from conventional antibiotics as they do not target the growth and the basal life processes of bacteria (Brooks and Brooks, 2014). Consequently, the development of resistance towards the drugs is minimized and they are expected to be effective against bacteria that are already resistant to conventional antibiotics. However, in chronic biofilm infections, mutations have often deleted the QS system (Ciofu et al., 2010). Many QSIs have been discovered (Fig. 2), which can either be derived from plants and fungal sources or chemically synthesized. However, except for azithromycin, no QSIs have so far successfully been tested in clinical trials for the treatment of chronic *P. aeruginosa* infections in CF (Bjarnsholt et al., 2013). One of the reasons is that most QSIs are poorly water soluble that limit their bioavailability and difficult to be formulated for inhalation administration (Nafee et al., 2014).

Earlier *in vivo* studies showed that the *P. aeruginosa* biofilms grown in the presence of synthetic furanone C-30, displayed an increased sensitivity to the tobramycin (Hentzer et al., 2003). Another study showed that the co-administration of tobramycin with furanone C-30 could

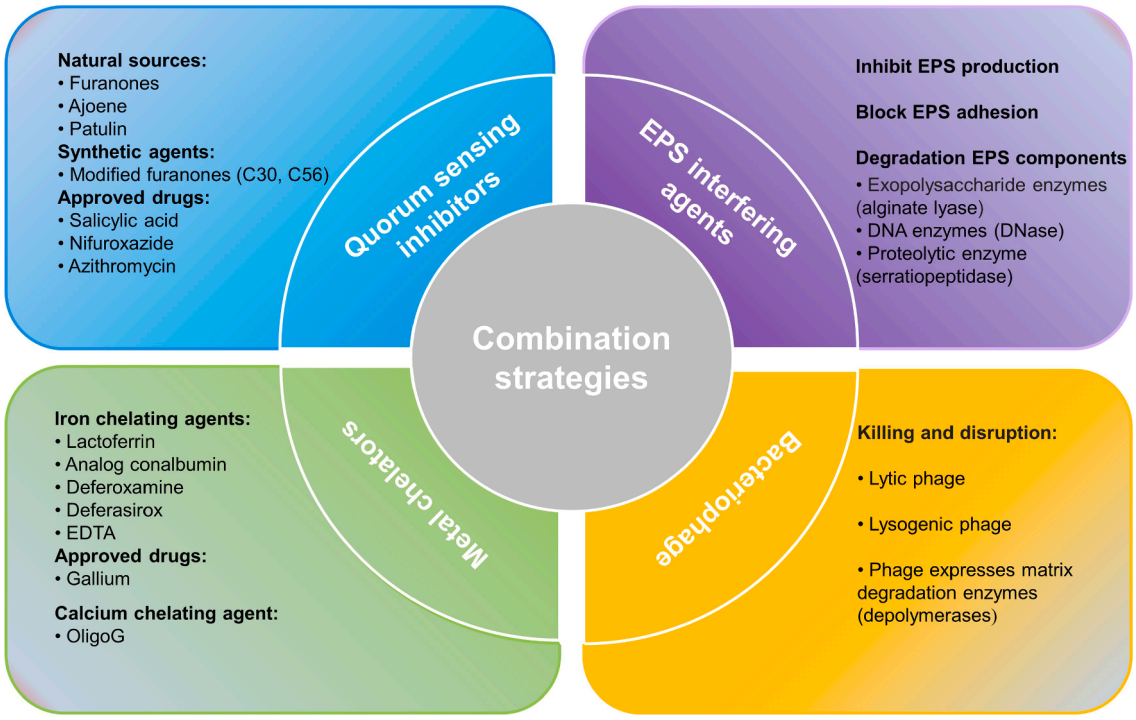

Fig. 2. Different combination therapy to combat biofilms.

efficiently eradicate the *P. aeruginosa* biofilm infection in a mouse model (Christensen et al., 2012). After the discovery of the furanone based QSIs, a flurry of other QSIs were discovered. Brackman *et al.* (Brackman et al., 2011) observed the synergistic effects of antibiotics and QSIs on the biofilms of *P. aeruginosa, Burkholderia cenocepacia*, and *Staphylococcus aureus*. Another potentially useful QSIs are garlic and garlic extracts (*i.e.,* allicin and ajoene) (Smyth et al., 2010). When tested in a trial of 26 CF patients, there was a greater decline in forced expiratory maneuver from baseline for patients in the placebo compared to the group treated with garlic. Although there was no significant effect of garlic compared to placebo in this pilot study, there was a suggestion of improvement with garlic (Smyth et al., 2010).

4.2. Combination of antibiotic and EPS interfering agents

The EPS matrix is important in maintaining biofilm structure and physiology, numerous strategies have been adopted to disrupt EPS (Gunn et al., 2016; Koo et al., 2017), rendering the bacteria to exist in their planktonic state, which is susceptible to the antibiotic treatment. EPS-targeting can be achieved by inhibiting EPS production, blocking EPS adhesins to surface, or degrading EPS in established biofilms (Fig. 2). EPS-degrading enzymes such as alginate lyase can reduce the alginate level in the EPS matrix of the biofilms, resulting in a reduced viscosity of the EPS matrix (Misagh et al., 2009a). The co-administration of the alginate lyase with gentamicin could display a greater eradication of the biofilms of mucoid *P. aeruginosa,* which were cultured in a condition, mimicking the CF of the respiratory tract (Alkawash et al., 2006). In addition, serratiopeptidase, a proteolytic enzyme, could also act as a biofilm disrupting agent by virtue of its proteolytic effect (Gupta et al., 2017). Levofloxacin at sub-MIC concentrations in combination with serratiopeptidase at lower concentrations showed significant eradication of preformed biofilm. Further, antibiofilm activity of serratiopeptidase was found to be proportional to biofilm forming capacity of *S. aureus* (Gupta et al., 2017). Moreover, an earlier study has demonstrated that the recombinant human DNase (*viz.*, dornase alfa) can degrade the neutrophils and microbial-derived DNA in patients with CF, thereby reducing the viscosity of the sputum (Manzenreiter et al., 2012). Repeated administration of DNase I-coated polymer nanoparticles encapsulating ciprofloxacin was able to reduce by 95% and then eradicate more than 99.8% of established biofilm in culture, outperforming the free-drug tested in the study (Baelo et al., 2015). In addition, the incorporation of DNase with levofloxacin could reduce the *P. aeruginosa* biofilms in culture (Islan et al., 2016). A Phase I study has been completed to evaluate the safety and tolerability of DNase in healthy volunteers (ClinicalTrials.gov identifier: NCT02605590).

4.3. Combination of antibiotic and metal-chelators

Iron plays a critical role in the development and maintenance of *P. aeruginosa* infections and it is utilized in both anabolic and signaling purposes (Hunter et al., 2013). The iron uptake and acquisition pathways have been identified as potential targets for antimicrobial agents (Banin et al., 2006; O'May et al., 2009). Currently, the associated therapeutic strategies are primarily focused on the blocking of ferric iron [Fe (III)] acquisition because Fe (III) is the most dominant and physiologically relevant form. Lactoferrin and its analog conalbumin (Hunter et al., 2013) and ethylene diamine tetra acetic acid (EDTA) (Banin et al., 2006) are employed to inhibit or eradicate the existing biofilms in culture. Moreover, the combination of tobramycin with deferoxamine or deferasirox reduced established biofilm biomass by approximately 90% and reduced viable bacteria by 7-log units (Moreau-Marquis et al., 2009). In addition, the FDA approved transition metal gallium, which is structurally analogous to iron could hinder the biofilm formation by disrupting the Fe (III) uptake and interfering the Fe signaling pathways (Koo et al., 2017). However, the oxidation state of iron was shown to be important (Hunter et al., 2013). Compared with Fe (III), ferrous iron [Fe (II)] was the primary form of bioavailable iron in the CF mucus, which also correlated with the severity of lung infections. This is possibly due to the generation of neutrophil-mediated superoxide and existence of hypoxic microenvironments in the late-stage of biofilm infections, reducing Fe (III) to its Fe (II) form (Hunter et al., 2013). Thus, Fe (II) chelation may provide a novel therapeutic strategy for CF lung infections.

In contrast to "broad-spectrum" chelating agents such as EDTA, the alginate oligomer (OligoG CF-5/20), a cationic chelator, exhibits low toxicity profile (Pritchard et al., 2015). The OligoG CF-5/20 is a low-molecular-weight oligosaccharide (Mw 3200 g/mol) and composed of L-guluronic acid and D-mannuronic acid with a degree of polymerization [DPn] of 16 (Pritchard et al., 2015). In a combination therapy with the cationic antibiotic, colistin, the OligoG CF-5/20 might chelate the divalent cations Ca^{2+}, and destroy the EPS of the biofilms (Pritchard et al., 2017). Consequently, the interactions of colistin with the biofilm matrix components is dramatically reduced, which increases its permeability to the biofilms and promotes the interactions between colistin and bacterial cell membrane, facilitating an improved drug permeation to the cytoplasm (Pritchard et al., 2017). The polymyxin B conjugated OligoG provides the potential benefits for the treatment of CF Gram-negative bacterial infections and minimizes antibiotic toxicity (Stokniene et al., 2019). The OligoG could also potentiate the antibiofilm activities of the macrolide antibiotics, such as erythromycin and tobramycin (Powell et al., 2018). Phase II studies of inhaled dry powder OligoG in adult patients with CF has completed (ClinicalTrials.gov identifier: NCT02157922 and NCT02453789). The OligoG CF-5/20 has been granted orphan status by the European Medicines Agency (EU/3/07/475) for the treatment of CF (Pritchard et al., 2015). Although the OligoG CF-5/20 with G content greater than 85% are known for its cationic chelating properties, the exact mechanism of action of its antibiofilm effects is still unclear (Powell et al., 2018). Additional research is needed to verify the impact of G content and molecular weight of OligoG on its antibacterial activities, and determine the precise molecular mechanisms of inhibition of microbial infections.

4.4. Combination of antibiotic and bacteriophage

The bacteriophages could replicate at the infection sites and efficiently kill the pathogens, while do not affect the symbiotics bacteria (Tian et al., 2021). Phage therapy utilizes obligatory lytic phages to kill its host, bacteria (Ng et al., 2021). Inhaled phage therapy was reported in the 1960s and its use was continuously improved in the Eastern European countries, particularly in Georgia, Russia and Poland (Hoe et al., 2013). Formulating phages into inhalable forms for pulmonary infections is a dual challenge requiring both aerosol performance and phage biochemical stability. Currently, phage research for respiratory infections has been focused on the delivery of liquid formulations and dry powder formulations (Chang et al., 2018). However, the development of phage-resistant bacteria (Chang et al., 2018) has led to repurposing of combination therapy using phages and antibiotics. Over the past few years, studies have repeatedly shown synergistic interaction between antibiotics and phages *in vitro*, which can potentially improve the clinical outcome in the treatment of pulmonary bacterial infections (Torres-Barcelo and Hochberg, 2016). Chaudhry *et al.* found that phage plus ceftazidime, ciprofloxacin or tobramycin could reduce the number of *in vitro* biofilm populations of *P. aeruginosa* PA14 (Chaudhry et al., 2017), and ciprofloxacin combined with phage treatment had the same effect (Henriksen et al., 2019). Moreover, Nouraldin *et al.* assessed the efficacy of phage and antibiotic combination against 15 clinical isolates on both planktonic and biofilm states (Nouraldin et al., 2016). Amikacin and meropenem showed synergistic effect with phages against planktonic cells, whereas amikacin with phages was effective against biofilms.

The number of phage therapy clinical trials on ClinicalTrials.gov is very limited and those with a focus on respiratory diseases are even rarer. Only one trial was identified (ClinicalTrials.gov identifier:

NCT01818206) and the aim was to assess the efficacy of a cocktail of 10 phages against *P. aeruginosa* in sputum samples from CF patients. Despite existing many challenges, inhaled combination of phage and antibiotic holds remarkable potential to help treat respiratory infections.

5. Nanotechnology-based drug delivery systems for combating respiratory biofilm infections

Drug delivery to the lungs by inhalation offers a targeted drug therapy for respiratory diseases (Guo et al., 2021). However, the therapeutic efficacy of inhaled drugs is limited by their rapid clearance in the lungs (Douafer et al., 2020). Nanotechnology-based drug delivery systems for antimicrobial agents have therefore been exploited to treat respiratory biofilm infections due to their many advantages over conventional antibiotic formulations, including controlled drug release, improved pharmacokinetic (PK) and pharmacodynamic (PD), targeting delivery, stabilization, modulated drug combination regimen etc. (Fig. 3). To date, lipid and polymer-based nanotechnology-based drug delivery systems may be the most exploited nanoparticles to deliver antimicrobial agents to treat respiratory biofilm infections due to their biocompatibility, biodegradability, safety profile and long history of usage (Sharma et al., 2021). Yet, the metal nanoparticles (*e.g.,* silver) and NO loaded nanoparticles have also been attempted and shown to be promising to combat biofilm infections as alternatives to classical antibiotics. The last but not the least, the nanoparticles exhibiting on-demand drug release upon specific stimuli are emerging and attracting more and more interest in this field with their 'smart' feature to achieve targeted and precise therapy.

5.1. Lipid-based nanoparticles

Liposomes are the only sustained-release strategy for pulmonary delivery that has been approved so. Antimicrobials have been successfully encapsulated in liposomes and some liposomal antimicrobials are very promising for biofilm treatment (Ding et al., 2021). Liposomes are composed of lipid bilayers that consist of phospholipids and cholesterol and can simultaneously encapsulate water-soluble and poorly water-soluble drugs (Fig. 4**a**). Liposomes loading antimicrobial drugs can fuse to the outer membranes of the bacteria owing to their membrane-like structure and release their cargos at high concentration into the cytoplasm of bacteria, bypassing the effects of efflux pump on drugs (Pelgrift and Friedman, 2013). Liposomes can be delivered to the human lungs by nebulization of a liposome suspension or as a dry powder (Li et al., 2018; Xu et al., 2019). However, liquid liposomal suspensions are sometimes associated with stability problems (Shetty et al., 2020). In order to overcome these issues, dry powder formulations have been developed for drug delivery to the lungs. A solid-state form of liposomal formulations containing ciprofloxacin nanocrystals retained stable at high relative humidity and controlled drug release (Khatib et al., 2020). Yu *et al.* also have developed the liposomal dry powder inhaler formulations consisting of ciprofloxacin and colistin (Yu et al., 2020). This study demonstrated that the combination liposomes could lower the transport capability of both drugs across the Calu-3 cell monolayer and accumulation in the cells. They can be prepared by spray-drying, spray-freeze-drying and freeze-drying followed by micronization (Elsayed and AbouGhaly, 2016; Yu et al., 2021).

The ability of the liposomes to penetrate into the biofilms has been investigated (Table 3). Neutrally charged liposomes in a certain size range have been shown to be able to effectively penetrate the expectorated CF sputum (Hadinoto and Cheow, 2014). It was reported that nebulized neutral liposomes of amikacin could treat the chronic *P. aeruginosa* infections in CF patients by effectively diffusing through the human respiratory mucus layer and penetrating into the biofilms, resulting in increased the local drug concentration around the bacteria (Meers et al., 2008). Despite anionic liposomes could reduce the interactions of the liposomes with mucus layer or EPS matrix, this might hinder their affinity towards the bacterial cells (Hadinoto and Cheow, 2014). Cationic liposomes could typically enhance the antimicrobial effects because of their superior interactions with the negatively charged bacterial cell membranes (Drulis-Kawa et al., 2009). However, the cationic liposomes could have strong interactions with the mucus and biofilm matrices, which retard the drug penetration and activity, as for example seen for tobramycin (Alipour et al., 2009). Therefore, neutral liposomes are considered to be better than the charged liposomes with respect to penetrating mucus layer and biofilms as well as interacting with the bacterial cells. Besides surface charge, the diameters of the liposomes were another important factor affecting biofilm penetration of liposomes. The ideal diameter for liposomes in biofilm-infection control ranges between 5 and 100–200 nm, but not exceeding 500 nm (Liu et al., 2019; Murgia et al., 2016). Although the mucus biofilms have significant clinical importance, their structure and the bacteria behaviors in these biofilms are not totally understood (Ho et al., 2019). It is notable that the mucus biofilms could exist for decades and cannot be completely eradicated.

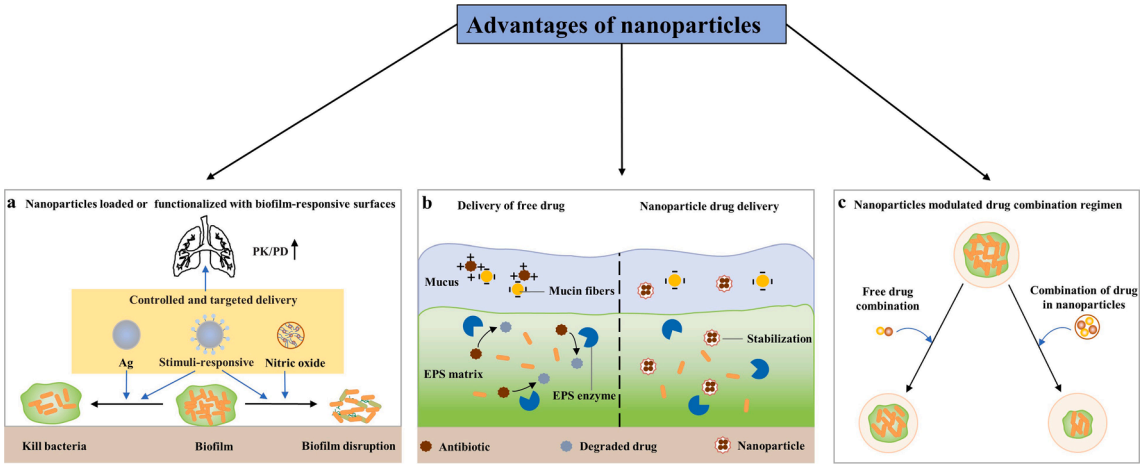

Fig. 3. The advantages of nanoparticles. a) Nanocarriers can be loaded or functionalized with biofilm-responsive surfaces that selectively target or control drug release within biofilms and improve the pharmacokinetic (PK) and pharmacodynamic (PD) profiles of antimicrobial agents. b) Nanoparticles can enhance the antibiotic penetration by reducing the interactions between the drug molecules and mucin as well as the EPS matrix and can protect drug from degradation by the enzymes present in the biofilm. c) Nanoparticles can be designed to efficiently deliver a combination of different functional actives, creating multifunctional carrier systems that also have been shown to produce improved efficacy over co-administration of the free drug combination.

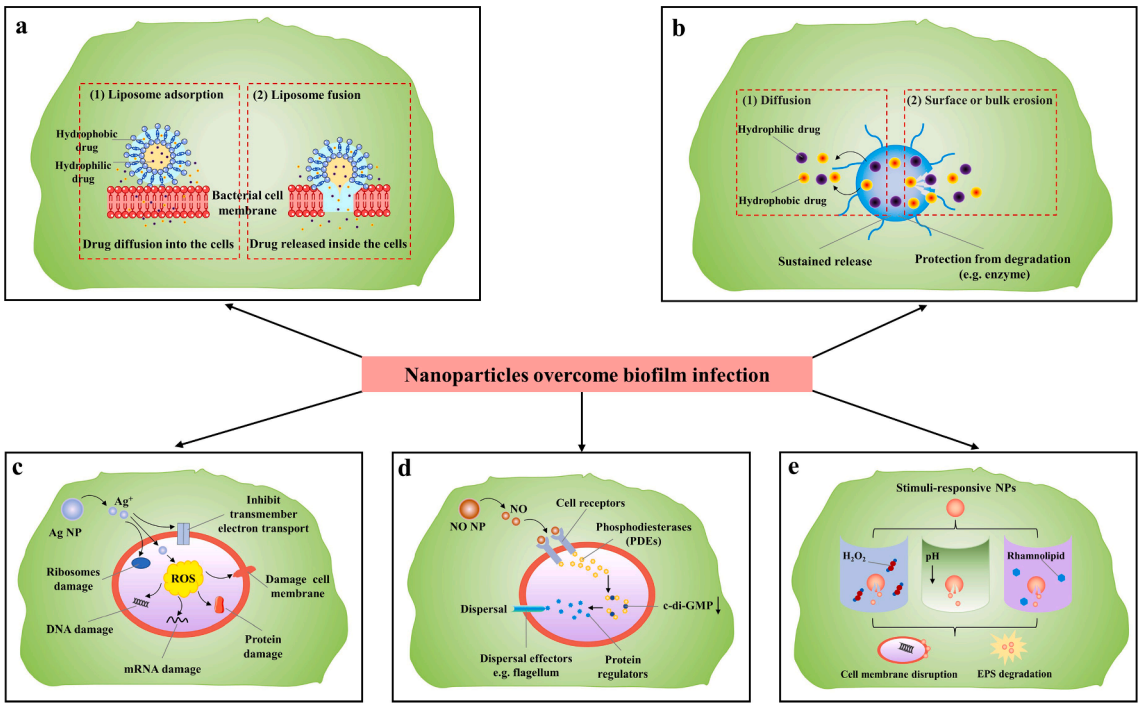

Fig. 4. Nanotechnology-based antimicrobials and delivery systems in biofilms. (a) Liposomes. (b) Polymer-based nanoparticles (NPs). (c) Ag and other metal NPs. (d) Nitric oxide (NO) NPs. (e) Stimuli-responsive NPs.

Table 3
A summary of representative delivery systems of the inhaled antibiotics for the treatment of respiratory biofilm infections.

Formulation	Major excipients	Antibiotics	Physical form	Size (nm)	Charge	Biofilm	Ref.
Liposomes	DPPC: Chol	Amikacin	LF	200–300	Neutral	*Non-tuberculous mycobacteria*	(Zhang et al., 2018)
	DPPC: Chol, DMPC: Chol	Polymyxin B, tobramycin	LF	300–450	Neutral	*P. aeruginosa*	(Misagh et al., 2009b)
	DMPC: Chol	Amikacin, gentamicin, tobramycin	LF	350–510	N/A	*P. aeruginosa*	(Misagh et al., 2009a)
	(DPPC, DDAB, DAP, Chol) combinations	Clarithromycin	LF	170–220	Neutral, Cationic, Anionic	*P. aeruginosa*	(Alhajlan et al., 2013)
	DPPC: Chol.	Amikacin	LF	300	N/A	*P. aeruginosa*	(Meers et al., 2008)
	DSPC: Chol	Tobramycin	DP	910	N/A	*P. aeruginosa*	(Alhariri and Omri, 2013)
	DPPC: DMPG, DSPC: DMPC	Tobramycin	LF	400	Neutral, Anionic	*P. aeruginosa*	(Beaulac et al., 1996)
	DMPC: Chol, DPPC: Chol, DSPC: Chol	Gentamycin	LF	400	Neutral	*P. aeruginosa*	(Mugabe et al., 2005)
Polymeric nanoparticles	PCL	Levofloxacin	DP	270	N/A	*E. coli*	(Kho et al., 2010)
	PLGA, PVA, PC	Levofloxacin	DP	240–300	Anionic	*P. aeruginosa*	(Cheow et al., 2011)
	PLGA, PVA, PL	Ciprofloxacin	DP	300	Cationic, Anionic	*P. aeruginosa*	(Baelo et al., 2015)
	PLGA, PCL	Ciprofloxacin, levofloxacin,	LF	170–240	N/A	*E. coli*	(Cheow et al., 2010a)
	PLGA, PC	Calcein (as a model)	LF	280	Anionic	*P. aeruginosa*	(Cheow et al., 2010b)

DPPC, dipalmitoylphosphatidylcholine; Chol, cholesterol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DDAB, didecyldimethylammonium bromide; DAP, 1,2-dioleoyl-3-dimethylammonium-propane; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); PCL, poly caprolactone; PLGA, poly (lactic-co-glycolic) acid; PVA, polyvinyl alcohol; PC, phosphatidylcholine; PL, poly (L-lysine); LF, liquid formulation; DP, dry powder; N/A, not available.

Besides liposomes, other lipid-based nanoparticles such as solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) could offer certain physical stability during the nebulization process for inhalation (Loira-Pastoriza et al., 2014). However, compared with liposomes, SLNs and NLC may be more toxic due to the presence of non-endogenous surfactants in their composition (Loira-Pastoriza et al., 2014). Currently, SLNs and NLCs are also investigated to treat pulmonary biofilm infections. It was reported that by incorporating liquid lipid in SLNs and NLCs the crystallinity of the lipid particles could be reduced, and the storage stability and the encapsulation efficiency could be

significantly improved (Wissing et al., 2004). A recent study showed that SLNs and NLCs containing levofloxacin exhibited a strong antibacterial activity against gram-positive and gram-negative microorganisms, which were also able to destroy the structure of biofilms of *P. aeruginosa* strain (Islan et al., 2016). Another study showed that colistin loaded NLCs were more effective than the pure drug in eradicating biofilms of *P. aeruginosa* in culture, which were attributed to the penetration ability of the encapsulated drug molecules to reach both the superficial and the deep regions of the biofilms (Eulalia et al., 2017). It was also shown that as compared to the free colistin, NLCs loading colistin exhibited a higher anti-biofilm activity against sensitive and resistant *P. aeruginosa* isolated from the sputum of CF patients (Sans-Serramitjana et al., 2016).

5.2. Polymer-based nanoparticles

Polymeric nanoparticles exhibit similar advantages of liposomes, such as mucus penetration and sustained drug release behavior (Ho et al., 2019). It was reported that polymeric nanoparticles could improve the residence time of the drug in the lung and protect the drug from the degradation (Fig. 4**b**). The biocompatible and biodegradable polymers, such as poly(lactic-co-glycolic) acid (PLGA), poly caprolactone (PCL), polyvinyl alcohol (PVA) have been widely used to deliver antibiotics into the lungs (Table 3). It should be noted that polymeric particles may cause lung toxicity due to the slow degradation rate of some polymers such as PLGA and their possible accumulation (Loira-Pastoriza et al., 2014).

One of the notable merits of polymeric nanoparticles may be their ability to provide prolonged drug release profile to maintain high concentration levels of antimicrobial agents at the site of the infections, which is considered to be critical with respect to improving the anti-biofilm effect (Forier et al., 2014). Cheow *et al.* (Cheow et al., 2010a, b) showed that levofloxacin and ciprofloxacin-loaded PLGA nanoparticles could exhibit biphasic drug release profile over six days, which were developed for the treatment of *Escherichia coli* biofilm cells infections. In another study, gentamicin loaded PLGA nanoparticles were tested against both planktonic and biofilm cultures of *P. aeruginosa* PA01 *in vitro* (Abdelghany et al., 2012). The PLGA nanoparticles provided a sustained release profile of gentamycin, showing a significantly effective killing effect against biofilms as compared to the free drug (Türeli et al., 2017). Similarly, ciprofloxacin counter-ion complex (sodium dodecyl sulfate)-loaded PLGA nanoparticles also exhibited sustained release profile and effective penetration of the tight mesh of biofilm/mucus, which were considered as promising nano-delivery systems to treat *P. aeruginosa* lung infections in CF patients (Abdelghany et al., 2012). However, prolonged retention time (weeks to months) of PLGA nanoparticles in the lungs raise also safety concerns. It is because PLGA could slowly degrade into acidic residues, *i.e.,* lactic acid and glycolic acid. The accumulation of acidic residues in the lungs might irritate the lung, causing cough, edema and others adverse effects (Wang et al., 2018). Hence, the biocompatibility of the polymeric nanoparticles for pulmonary administration to combat respiratory biofilm infection remains to be verified.

Coating nanoparticles with inert biocompatible polymers such as polyethylene glycol (PEG) represents the most popular strategy of surface functionalization. PEG is a neutral linear polyether, which has been approved by US food and drug administration (FDA) for pulmonary inhalation (Healy et al., 2014). It was found the PEGylated polystyrene nanoparticles could readily penetrate into the *P. aeruginosa* biofilms and mucus without interacting with their components, in contrast to the corresponding non-coated and charged counterparts (Forier et al., 2013). It was reported that PEG modification at the surface of negatively charged polystyrene nanoparticles with three different sizes (*i.e.,* 100, 200 and 500 nm) could diffuse across 10 μm-thick mucus (Suk et al., 2009). This can be attributed to the effect that PEG could avoid the interactions between the hydrophobic polystyrene nanoparticles and the hydrophobic domains of the mucin. In addition, the low molecular weight of PEG (2-5KDa) provided a no mucoadhesive coating on non-degradable latex beads, whereas high-molecular mass PEG (greater than10KDa) exhibited mucous membrane adhesion (Yang et al., 2011). The molecular weight and the surface density of PEG play an important role in mucus penetration of nanoparticles.

Natural polymers, such as chitosan or polylysine, can be used to prepare positively charged and antimicrobial nanoparticles (Liu et al., 2019). Among them, chitosan (linear polysaccharides derived from chitin) is the most commonly used (Pelgrift and Friedman, 2013). Positively charged chitosan chains can adsorb onto the negatively charged cell walls and plasma membranes of the microbial cells, causing aggregation and leakage of their intracellular content (Pelgrift and Friedman, 2013), inhibition of mRNA transcription and protein translation by binding to DNA (Friedman et al., 2013). Nanoparticles composed of chitosan possess a positively charged surface, enhancing their accumulation in biofilms (Liu et al., 2019). Moreover, chitosan could be chemically modified to treat biofilm infections. For example, aminated chitosan could exhibit an improved activity against biofilms (Kenawy et al., 2019). It was also reported that chitosan could be applied to provide a mucoadhesive coating on the PLGA nanoparticles (Chatterjee et al., 2017), which could increase the bioavailability and the performance of drugs both locally and systemically (Neves et al., 2011).

5.3. Metallic nanoparticles

Ag itself has only weak antimicrobial effects (Pelgrift and Friedman, 2013). The antibacterial potentials of Ag nanoparticles are mostly due to their dissolution and release of Ag^+ ions, which can interact with the cell membrane and subsequently generate ROS and cell lysis (Fig. 4**c**). Studies have shown that Ag nanoparticles not only have antibacterial effect on planktonic bacteria (Dakal et al., 2016), but also have anti-biofilm effect (Singh et al., 2019). However, the mechanism by which Ag nanoparticles penetrate and kill biofilms remains unclear. The antimicrobial/antibiofilm activities of Ag nanoparticles are dependent upon their size, and small Ag nanoparticles show high bacteriostatic ability (Knetsch and Koole, 2011). Due to their high surface energy, the Ag nanoparticles are easily aggregated into large particles, which might weaken their antibiofilm capacity (Pelgrift and Friedman, 2013). Therefore, surface functionalization before application is important. Natural and environmentally benign compounds like β-cyclodextrin and polyphenols are used to stabilize colloidal Ag nanoparticles by coupling through hydrogen bonding (Liu et al., 2019). In addition, antimicrobials can be used synergistically with Ag nanoparticles to enhance bacterial killing by chelating antimicrobial carboxyl/amino groups on the Ag nanoparticles (Liu et al., 2019).

In addition, the metallic copper (Cu) and gold (Au) were also encapsulated in nanoparticles, which showed a weaker antibacterial effect than the Ag nanoparticle (Pelgrift and Friedman, 2013). The magnesium halogen (MgF_2) (Blecher et al., 2011), zinc oxide (ZnO) (Pelgrift and Friedman, 2013) and ferroferric oxide (Fe_3O_4) (Beyth et al., 2015) nanoparticles could also inhibit the growth of the bacteria and biofilm formation. Despite of the great potential of the metal nanoparticles, there are relatively limited clinical application in lung biofilm infection, as the related nanotechnology requires a complex characterization and analysis. In addition, the scaling up of nano-delivery systems is also challenging. Last but not the least, the long-term administration of the metal nanoparticles for the lung infections and their inhalation administration are not preferred due to their non-biodegradable nature.

5.4. Nitric oxide (NO) nanoparticles

NO is a signaling molecule found in both prokaryotic and eukaryotic systems, which can reduce bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) level by increasing the phosphodiesterases (PDEs)

sensing (Rumbaugh and Sauer, 2020), ultimately mediate dispersal of *P. aeruginosa* biofilm and exert antibacterial effects (Fig. 4**d**). Although the regulation of the c-di-GMP is complicated, this signaling pathway provides an attractive therapeutic strategy for the bacteria eradication. In a randomized clinical trial on 12 patients, the inhalation of 10 ppm of NO caused a significant reduction in *P. aeruginosa* biofilm aggregates as compared to the placebo therapy after 7 days of treatment (Howlin et al., 2017). Hetrick *et al.* showed that the NO nanoparticles made of silica could kill bacteria, including *P. aeruginosa, S. aureus* and other bacteria existing in the biofilms (Hetrick et al., 2009). In addition, NO could react with superoxide (O^{2-}) to form reactive nitrogen oxide intermediates (RNOS), which can directly damage the proteins, DNA, DNA repairing enzymes and membrane of bacterial (Pelgrift and Friedman, 2013). NO nanoparticles exhibited a broad-spectrum antibacterial potential and could inhibit the growth of antibiotic-resistant bacteria, such as *P. aeruginosa* (Pelgrift and Friedman, 2013). Currently, there is no direct evidence showing that the use of exogenous NO could lead to bacterial resistance, which might be due to its multiple antibacterial mechanisms. Limited studies in humans subjects shows that topically delivered NO is clinically useful and no more toxic than currently available antimicrobials (Schairer et al., 2012). It could be speculated that NO-based nanoparticles might be promising because it is an inexpensive, simple to synthesize, shelf stable and nontoxic, which could provide sustained release of NO to exert the killing effect.

5.5. Stimuli-responsive nanoparticles

Nanotechnology-based drug delivery systems with stimuli-triggered drug release behavior have attracted enormous interest in the field (Fig. 4**e**). It is because they could elute their cargos in the close proximity of the biofilm bacteria. They were described as 'smart' and considered as a promising approach in achieving a precise targeted therapy by increasing the local antibiotic concentration in the biofilms and reducing systemic side effects. Several stimuli factors including oxygen concentrations, pH, virulence factors and proteins/enzymes are exploited to trigger the release of antibacterial agents (Benoit and Koo, 2016). However, these mechanisms have not been reported yet in clinical research.

The biofilm bacteria growing in the CF airways are exposed to ROS liberated from PMNs (Mauch et al., 2018). This could lead to the development of oxidative stress, which could cause an enhanced adaptability of bacteria in the biofilm communities (Boles and Singh, 2008). Inspired by the aberrantly increased ROS level at the disease sites, a large number of ROS-triggerable compounds, materials, and drug delivery systems have been developed for the biofilm therapy (Zhang et al., 2017). A ROS-responsive material, *i.e.,* 4-(hydroxymethyl) phenylboronic acid pinacol ester-modified α-cyclodextrin, was employed to encapsulate moxifloxacin (Wang et al., 2019). This resulting ROS-responsive moxifloxacin-loaded nanoparticles could overcome the mucus barrier, control drug release in the presence of 0.5 mM H_2O_2, and improve the targeting capability of nanoparticles for the treatment of pulmonary bacterial infections (Wang et al., 2019).

The biofilms display an acidic microenvironment (pH ~ 4.5 or even lower in some cases) (Benoit and Koo, 2016). The acidic environment is caused by the anaerobic fermentation (Benoit and Koo, 2016) of the microorganisms and activated PMNs (Benoit and Koo, 2016; Jensen et al., 2017). Some aminoglycoside antibiotics are known to lose their activity in acidic environment, *e.g.,* under CF conditions (Benoit and Koo, 2016; Simmen and Blaser, 1993). Researchers developed pH-responsive nanoparticles, which were stable under physiologic pH conditions but degrade or disrupt under acidic pH conditions to release the active drug molecules. Some commonly used pH-responsive functional groups include esters, ketals, acetals, and anhydrides etc., which could be utilized to design pH-responsive nanoparticles encapsulating antibiotics (Benoit and Koo, 2016).

Biosurfactant rhamnolipid, virulence factors, which was shown to be important for biofilm formation (Boles et al., 2005). Recent study showed that rhamnolipid could trigger the release of cargos in lipid-polymer hybrid nanoparticles consisting of PLGA cores and phosphatidylcholine shells, but only suitable for certain classes of encapsulated molecules (Cheow and Hadinoto, 2012). For example, the lipid-polymer hybrid nanoparticles were used to encapsulate levofloxacin and ofloxacin and both of these drugs could rapidly be released from the nanoparticles in the absence of rhamnolipids, which was attributed to the fact that both drugs are able to pass lipid membranes. However, a water-soluble dye, calcein with a low permeability to the lipid membranes, showed no significant release of calcein in the absence of rhamnolipids. With an addition of rhamnolipids, calcein could be rapidly released. In another study, Meers *et al.* showed that rhamnolipid could trigger the release of amikacin from the inhaled DPPC: Chol liposomes for more than 8 h, due to its surfactant-like property, which could successfully penetrate to the biofilms in *in vivo* study (Meers et al., 2008).

6. Conclusion

Antibiotic management is a primary treatment regimen in the clinic to combat respiratory biofilm infections. As a common clinical practice, antibiotics are given *via* inhalation to treat chronic respiratory infections with biofilms besides systemic administration by means of injection/oral treatment. The rationale behind this dosing regimen is because high local concentrations of antibiotics can be achieved *via* inhalation. While inhalation delivery of antibiotics provides certain clinical benefits, the eradication of pathogens within biofilms has not been very successful. An efficient treatment of biofilm infections needs a well-established multidisciplinary approach, which may include the removal of the infected foreign bodies, selection of biofilm-sensitive antibiotics, administration of anti-quorum sensing or biofilm dispersal agents, and their combinations, besides the systemic or topical antibiotic administration in high dose. More recently, nanotechnology-based drug delivery systems have been exploited to combat biofilm infections. Despite of lacking the clinical evidences, the flexibility of the nanotechnology-based drug delivery systems may offer promising opportunities to overcome bacterial resistance and improve the treatment of respiratory biofilm infections.

7. Future perspective

The formation of biofilm is a key strategy for microorganisms to survive and plays a major role in the persistence of bacterial infections as the cells in the biofilms are able to evade host immune defenses and increase antibiotic resistance. An effective therapeutic strategy against respiratory bacterial biofilm infections may be exploiting drug combinations. The simultaneous delivery of multiple antibiotics, and also combing antibiotics and non-antibiotic substance such as QSIs, EPS interfering agents, metal-chelators, bacteriophage etc. could enhance the antimicrobial efficacy of the antibiotics at a reduced dose employed. In addition, the development of bacterial resistance could be circumvented by this combinational therapy. Recently, various nanoparticle-based drug delivery systems composed of lipids, polymers, metals, and NO have shown advantages over conventional antibiotic management to combat respiratory biofilm infections by providing high local concentrations of antibiotics to the site of infection, enhancing the selectivity and sensitivity, and suppression of the resistance development. Moreover, 'smart' nanotechnology-based drug delivery systems functionalized by stimuli-responsive moieties, have shown 'on-demand' release or activation of bioactive agents triggered by pathogenic microenvironments (*e.g.,* pH, hypoxia, and virulence factors), which may further enhance the treatment efficiency of respiratory bacterial biofilm infections. Despite creating promising concepts with these advanced pharmaceutical technologies, the optimization of the pharmaceutical formulation characteristics and the dosing regimen are still required, in particular the drug loading capacity, *in vivo* respiratory biocompatibility

at high doses, and the exposure of antibiotics to the biofilm. Most current research are based on *in vitro* observations, with many failing to progress to *in vivo* studies and even fewer to clinical application. The poor predictability of the *in vitro* biofilm models also hinders the development of the treatment strategies of the respiratory biofilm infections. It warrants collecting appropriate clinical samples, for antibiotic susceptibility testing and for improvement of laboratory reports of biofilm findings in the clinical microbiology laboratory. The interested reader is referred to a recent review article presenting all the pulmonary *P. aeruginosa* infectious models focusing on macrolide therapy (Thomsen et al., 2021). In addition, the future research should focus on achieving a maximal efficacy and specificity with minimal toxicity and long-term effects to develop low-cost and practical formulations for clinical use.

CRediT authorship contribution statement

Li Zhang: Writing – original draft. **Hriday Bera:** Writing – review & editing. **Hengzhuang Wang:** Writing – review & editing. **Junwei Wang:** Writing – review & editing. **Yi Guo:** Writing – review & editing. **Changzhi Shi:** Writing – review & editing. **Dongmei Cun:** Writing – review & editing. **Claus Moser:** Writing – review & editing. **Niels Høiby:** Writing – review & editing. **Mingshi Yang:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

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References

Aanaes, K., Eickhardt, S., Johansen, H.K., von Buchwald, C., Skov, M., Høiby, N., Bjarnsholt, T., 2015. Sinus biofilms in patients with cystic fibrosis: is adjusted eradication therapy needed? Eur. Arch. Otorhinolaryngol. 272 (9), 2291–2297. https://doi.org/10.1007/s00405-014-3322-x.

Abdelghany, S.M., Quinn, D.J., Ingram, R.J., Gilmore, B., Donnelly, R.F., Taggart, C.C., Scott, C.J., 2012. Gentamicin-loaded nanoparticles show improved antimicrobial effects towards infection. Int. J. Nanomedicine. 7, 4053–4063. https://doi.org/10.2147/IJN.S34341.

Alanin, M.C., Johansen, H.K., Aanaes, K., Høiby, N., Pressler, T., Skov, M., Nielsen, K.G., von Buchwald, C., 2015. Simultaneous sinus and lung infections in patients with primary ciliary dyskinesia. Acta Otolaryngol. 135 (1), 58–63. https://doi.org/10.3109/00016489.2014.962185.

Alhajlan, M., Alhariri, M., Omri, A., 2013. Efficacy and safety of liposomal clarithromycin and its effect on *Pseudomonas aeruginosa* virulence factors. Antimicrob. Agents Chemother. 57 (6), 2694–2704. https://doi.org/10.1128/AAC.00235-13.

Alhariri, M., Omri, A., 2013. Efficacy of liposomal bismuth-ethanedithiol-loaded tobramycin after intratracheal administration in rats with pulmonary *Pseudomonas aeruginosa* infection. Antimicrob. Agents Chemother. 57 (1), 569–578. https://doi.org/10.1128/AAC.01634-12.

Alipour, M., Suntres, Z.E., Halwani, M., Azghani, A.O., Omri, A., 2009. Activity and interactions of liposomal antibiotics in presence of polyanions and sputum of patients with cystic fibrosis. Plos One. 4, e5724. https://doi.org/10.1371/journal.pone.0005724.

Alkawash, M.A., Soothill, J.S., Schiller, N.L., 2006. Alginate lyase enhances antibiotic killing of mucoid *Pseudomonas aeruginosa* in biofilms. APMIS. 114 (2), 131–138. https://doi.org/10.1111/j.1600-0463.2006.apm_356.x.

Anwar, H., Dasgupta, M., Lam, K., Costerton, J.W., 1989. Tobramycin resistance of mucoid *Pseudomonas aeruginosa* biofilm grown under iron limitation. J. Antimicrob. Chemother. 24 (5), 647–655. https://doi.org/10.1093/jac/24.5.647.

Baelo, A., Levato, R., Julian, E., Crespo, A., Astola, J., Gavalda, J., Engel, E., Angel Mateos-Timoneda, M., Torrents, E., 2015. Disassembling bacterial extracellular matrix with DNase-coated nanoparticles to enhance antibiotic delivery in biofilm infections. J. Control Release. 209, 150–158. https://doi.org/10.1016/j.jconrel.2015.04.028.

Bagge, N., Hentzer, M., Andersen, J.B., Ciofu, O., Givskov, M., Hoiby, N., 2004. Dynamics and spatial distribution of beta-lactamase expression in *Pseudomonas aeruginosa* biofilms. Antimicrob. Agents Chemother. 48, 1168–1174. https://doi.org/10.1128/aac.48.4.1168-1174.2004.

Ballmann, M., Smyth, A., Geller, D.E., 2011. Therapeutic approaches to chronic cystic fibrosis respiratory infections with available, emerging aerosolized antibiotics. Respiratory Medicine. 105, S2–S8. https://doi.org/10.1016/s0954-6111(11)70021-x.

Banin, E., Brady, K.M., Greenberg, E.P., 2006. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. Appl. Environ. Microbiol. 72 (3), 2064–2069. https://doi.org/10.1128/AEM.72.3.2064-2069.2006.

Beaulac, C., Clément-Major, S., Hawari, J., Lagacé, J., 1996. Eradication of mucoid *Pseudomonas aeruginosa* with fluid liposome-encapsulated tobramycin in an animal model of chronic pulmonary infection. Antimicrob. Agents Chemother. 40 (3), 665–669. https://doi.org/10.1128/AAC.40.3.665.

Benoit, D.SW., Koo, H., 2016. Targeted, triggered drug delivery to tumor and biofilm microenvironments. Nanomedicine. 11 (8), 873–879. https://doi.org/10.2217/nnm-2016-0014.

Beyth, N., Houri-Haddad, Y., Domb, A., Khan, W., Hazan, R., 2015. Alternative antimicrobial approach: Nano-antimicrobial materials. Evid. Based Complement Alternat. Med. 2015, 1–16. https://doi.org/10.1155/2015/246012.

Bjarnsholt, T., Ciofu, O., Molin, S., Givskov, M., Høiby, N., 2013. Applying insights from biofilm biology to drug development - can a new approach be developed? Nat. Rev. Drug Discov. 12 (10), 791–808. https://doi.org/10.1038/nrd4000.

Bjarnsholt, T., Jensen, P.Ø., Fiandaca, M.J., Pedersen, J., Hansen, C.R., Andersen, C.B., Pressler, T., Givskov, M., Høiby, N., 2010. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr. Pulmonol. 44 (6), 547–558. https://doi.org/10.1002/ppul.21011.

Blecher, K., Nasir, A., Friedman, A., 2011. The growing role of nanotechnology in combating infectious disease. Virulence. 2 (5), 395–401. https://doi.org/10.4161/viru.2.5.17035.

Boles, B.R., Singh, P.K., 2008. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. Proc. Natl. Acad. Sci. USA 105 (34), 12503–12508. https://doi.org/10.1073/pnas.0801499105.

Boles, B.R., Thoendel, M., Singh, P.K., 2005. Rhamnolipids mediate detachment of Pseudomonas aeruginosa from biofilms. Mol. Microbiol. 57, 1210–1223. https://doi.org/10.1111/j.1365-2958.2005.04743.x.

Borriello, G., Werner, E., Roe, F., Kim, A.M., Ehrlich, G.D., Stewart, P.S., 2004. Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. Antimicrob. Agents Chemother. 48 (7), 2659–2664. https://doi.org/10.1128/AAC.48.7.2659-2664.2004.

Brackman, G., Cos, P., Maes, L., Nelis, H.J., Coenye, T., 2011. Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo*. Antimicrob. Agents Chemother. 55 (6), 2655–2661. https://doi.org/10.1128/AAC.00045-11.

Brooks, B., Brooks, A.E., 2014. Therapeutic strategies to combat antibiotic resistance. Adv. Drug Deliv. Rev. 78, 14–27. https://doi.org/10.1016/j.addr.2014.10.027.

Cao, B., Christophersen, L., Kolpen, M., Jensen, P.O., Sneppen, K., Hoiby, N., Moser, C., Sams, T., 2016. Diffusion retardation by binding of tobramycin in an alginate biofilm model. Plos One. 11, e0153616. http://doi.org/10.1371/journal.pone.0153616.

Cao, B., Christophersen, L., Thomsen, K., Sonderholm, M., Bjarnsholt, T., Jensen, P.O., Hoiby, N., Moser, C., 2015. Antibiotic penetration and bacterial killing in a *Pseudomonas aeruginosa* biofilm model. J. Antimicrob. Chemother. 70, 2057–2063. https://doi.org/10.1093/jac/dkv058.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D., Buret, A., 1999. The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J. Clin. Microbiol. 37 (6), 1771–1776. https://doi.org/10.1128/JCM.37.6.1771-1776.1999.

Chang, R.Y.K., Wallin, M., Lin, Y., Leung, S.S.Y., Wang, H., Morales, S., Chan, H.K., 2018. Phage therapy for respiratory infections. Adv. Drug Deliv. Rev. 133, 76–86. https://doi.org/10.1016/j.addr.2018.08.001.

Chatterjee, B., Amalina, N., Sengupta, P., Mandal, U.K., 2017. Mucoadhesive polymers and their mode of action: a recent update. J. Pharm. Sci. 7, 195–203. https://doi.org/10.7324/JAPS.2017.70533.

Chaudhry, W.N., Concepcion-Acevedo, J., Park, T., Andleeb, S., Bull, J.J., Levin, B.R., 2017. Synergy and order effects of antibiotics and phages in killing *Pseudomonas aeruginosa* biofilms. Plos One. 12, e0168615. http://doi.org/10.1371/journal.pone.0168615.

Cheow, W.S., Chang, M.W., Hadinoto, K., 2010a. Antibacterial efficacy of inhalable antibiotic-encapsulated biodegradable polymeric nanoparticles against *E. coli* biofilm cells. J. Biomed. Nanotechnol. 6, 391–403. https://doi.org/10.1166/jbn.2010.1116.

Cheow, W.S., Chang, M.W., Hadinoto, K., 2010b. Antibacterial efficacy of inhalable levofloxacin-loaded polymeric nanoparticles against *E. coli* biofilm cells: the effect of antibiotic release profile. Pharm Res. 27 (8), 1597–1609. https://doi.org/10.1007/s11095-010-0142-6.

Cheow, W.S., Chang, M.W., Hadinoto, K., 2011. The roles of lipid in anti-biofilm efficacy of lipid–polymer hybrid nanoparticles encapsulating antibiotics. Colloid Surf. A. 389 (1-3), 158–165. https://doi.org/10.1016/j.colsurfa.2011.08.035.

Cheow, W.S., Hadinoto, K., 2012. Lipid-polymer hybrid nanoparticles with rhamnolipid-triggered release capabilities as anti-biofilm drug delivery vehicles. Particuology. 10 (3), 327–333. https://doi.org/10.1016/j.partic.2011.08.007.

Chiang, W.-C., Nilsson, M., Jensen, P.Ø., Høiby, N., Nielsen, T.E., Givskov, M., Tolker-Nielsen, T., 2013. Extracellular DNA shields against aminoglycosides in *Pseudomonas aeruginosa* biofilms. Antimicrob. Agents Chemother. 57 (5), 2352–2361.

Christensen, L.D., van Gennip, M., Jakobsen, T.H., Alhede, M., Hougen, H.P., Hoiby, N., Bjarnsholt, T., Givskov, M., 2012. Synergistic antibacterial efficacy of early combination treatment with tobramycin and quorum-sensing inhibitors against *Pseudomonas aeruginosa* in an intraperitoneal foreign-body infection mouse model. J Antimicrob Chemother. 67 (5), 1198–1206. https://doi.org/10.1093/jac/dks002.

Christophersen, L., Schwartz, F.A., Lerche, C.J., Svanekjær, T., Kragh, K.N., Laulund, A. S., Thomsen, K., Henneberg, K.-Å., Sams, T., Høiby, N., Moser, C., 2020. In vivo demonstration of *Pseudomonas aeruginosa* biofilms as independent pharmacological microcompartments. J. Cyst. Fibros. 19 (6), 996–1003. https://doi.org/10.1016/j.jcf.2020.01.009.

Ciofu, O., Lee, B., Johannesson, M., Hermansen, N.O., Meyer, P., Hoiby, N., Scandinavian Cystic Fibrosis, S., 2008. Investigation of the algT operon sequence in mucoid and non-mucoid *Pseudomonas aeruginosa* isolates from 115 Scandinavian patients with cystic fibrosis and in 88 in vitro non-mucoid revertants. Microbiol-SGM. 154, 103–113. http://doi.org/10.1099/mic.0.2007/010421-0.

Ciofu, O., Mandsberg, L.F., Bjarnsholt, T., Wassermann, T., Hoiby, N., 2010. Genetic adaptation of *Pseudomonas aeruginosa* during chronic lung infection of patients with cystic fibrosis: strong and weak mutators with heterogeneous genetic backgrounds emerge in mucA and/or lasR mutants. Microbiol-SGM. 156, 1108–1119. https://doi.org/10.1099/mic.0.033993-0.

Ciofu, O., Tolker-Nielsen, T., Jensen, P.O., Wang, H.Z., Hoiby, N., 2015. Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. Adv. Drug Deliv. Rev. 85, 7–23. https://doi.org/10.1016/j.addr.2014.11.017.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J., 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41 (1), 435–464. https://doi.org/10.1146/annurev.mi.41.100187.002251.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. Science. 284 (5418), 1318–1322.

Cowley, E.S., Kopf, S.H., LaRiviere, A., Ziebis, W., Newman, D.K., Spormann, A., Relman, D.A., 2015. Pediatric cystic fibrosis sputum can be chemically dynamic, anoxic, and extremely reduced due to hydrogen sulfide formation. MBio. 6 (4) https://doi.org/10.1128/mBio.00767-15.

Dakal, T.C., Kumar, A., Majumdar, R.S., Yadav, V., 2016. Mechanistic basis of antimicrobial actions of silver nanoparticles. Front Microbiol. 7 https://doi.org/10.7389/fmicb.2016.01831.

Damron, F.H., Qiu, D., Yu, H.D., 2009. The *Pseudomonas aeruginosa* sensor kinase kinB negatively controls alginate production through AlgW-dependent MucA proteolysis. J. Bacteriol. 191 (7), 2285–2295.

De Soyza, A., Aksamit, T., Bandel, T.-J., Criollo, M., Elborn, J.S., Operschall, E., Polverino, E., Roth, K., Winthrop, K.L., Wilson, R., 2018. RESPIRE 1: a phase III placebo-controlled randomised trial of ciprofloxacin dry powder for inhalation in non-cystic fibrosis bronchiectasis. Eur. Respir. J. 51 (1), 1702052. https://doi.org/10.1183/13993003.02052-201710.1183/13993003.02052-2017.Supp110.1183/13993003.02052-2017.Supp2.

Deppisch, C., Herrmann, G., Graepler-Mainka, U., Wirtz, H., Heyder, S., Engel, C., Marschal, M., Miller, C.C., Riethmüller, J., 2016. Gaseous nitric oxide to treat antibiotic resistant bacterial and fungal lung infections in patients with cystic fibrosis: a phase I clinical study. Infection. 44 (4), 513–520. https://doi.org/10.1007/s15010-016-0879-x.

Ding, L.i., Wang, J., Cai, S., Smyth, H., Cui, Z., 2021. Pulmonary biofilm-based chronic infections and inhaled treatment strategies. Int. J. Pharm. 604, 120768. https://doi.org/10.1016/j.ijpharm.2021.120768.

Douafer, H., Andrieu, V., Brunel, J.M., 2020. Scope and limitations on aerosol drug delivery for the treatment of infectious respiratory diseases. J. Control Release. 325, 276–292. https://doi.org/10.1016/j.jconrel.2020.07.002.

Drulis-Kawa, Z., Dorotkiewicz-Jach, A., Gubernator, J., Gula, G., Bocer, T., Doroszkiewicz, W., 2009. The interaction between *Pseudomonas aeruginosa* cells and cationic PC:Chol:DOTAP liposomal vesicles versus outer-membrane structure and envelope properties of bacterial cell. Int. J. Pharm. 367 (1-2), 211–219. https://doi.org/10.1016/j.ijpharm.2008.09.043.

Duncan, G.A., Jung, J., Joseph, A., Thaxton, A.L., West, N.E., Boyle, M.P., Hanes, J., Suk, J.S., 2016. Microstructural alterations of sputum in cystic fibrosis lung disease. JCI Insight. 1 (18) https://doi.org/10.1172/jci.insight.8819810.1172/jci.insight.88198DS1.

Elsayed, I., AbouGhaly, M.H.H., 2016. Inhalable nanocomposite microparticles: preparation, characterization and factors affecting formulation. Expert Opin. Drug Discov. 13 (2), 207–222. https://doi.org/10.1517/17425247.2016.1102224.

Eulalia, S.S., Marta, J., Luis, P.J., Teresa, V., Miguel, V., 2017. Determination of the spatiotemporal dependence of *Pseudomonas aeruginosa* biofilm viability after treatment with NLC-colistin. Int. J. Nanomedicine. 12, 4409–4413. https://doi.org/10.2147/IJN.S138763.

Fernández-Barat, L., Ciofu, O., Kragh, K.N., Pressler, T., Johansen, U., Motos, A., Torres, A., Hoiby, N., 2017. Phenotypic shift in *Pseudomonas aeruginosa* populations from cystic fibrosis lungs after 2-week antipseudomonal treatment. J. Cyst. Fibros. 16 (2), 222–229. https://doi.org/10.1016/j.jcf.2016.08.005.

Folkesson, A., Jelsbak, L., Yang, L., Johansen, H.K., Ciofu, O., Høiby, N., Molin, S., 2012. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat. Rev. Microbiol. 10 (12), 841–851. https://doi.org/10.1038/nrmicro2907.

Forier, K., Messiaen, A.-S., Raemdonck, K., Deschout, H., Rejman, J., De Baets, F., Nelis, H., De Smedt, S.C., Demeester, J., Coenye, T., Braeckmans, K., 2013. Transport of nanoparticles in cystic fibrosis sputum and bacterial biofilms by single-particle tracking microscopy. Nanomedicine. 8 (6), 935–949.

Forier, K., Raemdonck, K., Smedt, S.D., Demeester, J., Coenye, T., Braeckmans, K., 2014. Lipid and polymer nanoparticles for drug delivery to bacterial biofilms. J. Control Release. 190, 607–623. https://doi.org/10.1016/j.jconrel.2014.03.055.

Friedman, A.J., Phan, J., Schairer, D.O., Champer, J., Qin, M., Pirouz, A., Blecher-Paz, K., Oren, A., Liu, P.T., Modlin, R.L., Kim, J., 2013. Antimicrobial and anti-inflammatory activity of chitosan-alginate nanoparticles: a targeted therapy for cutaneous pathogens. J. Invest. Dermatol. 133 (5), 1231–1239. https://doi.org/10.1038/jid.2012.399.

Gunn, J.S., Bakaletz, L.O., Wozniak, D.J., 2016. What's on the outside matters: the role of the extracellular polymeric substance of Gram-negative biofilms in evading host immunity and as a target for therapeutic intervention. J. Biol. Chem. 291 (24), 12538–12546. https://doi.org/10.1074/jbc.R115.707547.

Guo, Y.i., Bera, H., Shi, C., Zhang, L.i., Cun, D., Yang, M., 2021. Pharmaceutical strategies to extend pulmonary exposure of inhaled medicines. Acta Pharmaceutica Sinica B. 11 (8), 2565–2584.

Gupta, P.V., Nirwane, A.M., Belubbi, T., Nagarsenker, M.S., 2017. Pulmonary delivery of synergistic combination of fluoroquinolone antibiotic complemented with proteolytic enzyme: a novel antimicrobial and antibiofilm strategy. Nanomedicine. 13 (7), 2371–2384. https://doi.org/10.1016/j.nano.2017.06.011.

Hadinoto, K., Cheow, W.S., 2014. Nano-antibiotics in chronic lung infection therapy against *Pseudomonas aeruginosa*. Colloids Surf. B Biointerfaces. 116, 772–785. https://doi.org/10.1016/j.colsurfb.2014.02.032.

Hassett, D.J., Cuppoletti, J., Trapnell, B., Lymar, S.V., Rowe, J.J., Sun Yoon, S., Hilliard, G.M., Parvatiyar, K., Kamani, M.C., Wozniak, D.J., Hwang, S.-H., McDermott, T.R., Ochsner, U.A., 2002. Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Adv. Drug Deliv. Rev. 54 (11), 1425–1443. https://doi.org/10.1016/S0169-409X(02)00152-7.

Hassett, D.J., Korfhagen, T.R., Irvin, R.T., Schurr, M.J., Sauer, K., Lau, G.W., Sutton, M. D., Yu, H., Hoiby, N., 2010. *Pseudomonas aeruginosa* biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies. Expert Opin. Ther. Targets. 14 (2), 117–130. https://doi.org/10.1517/14728220903454988.

Healy, A.M., Amaro, M.I., Paluch, K.J., Tajber, L., 2014. Dry powders for oral inhalation free of lactose carrier particles. Adv. Drug Deliv. Rev. 75, 32–52. https://doi.org/10.1016/j.addr.2014.04.005.

Heijerman, H., Westerman, E., Conway, S., Touw, D., Doring, G., Consensus Working, G., 2009. Inhaled medication and inhalation devices for lung disease in patients with cystic fibrosis: A European consensus. J. Cyst. Fibros. 8, 295–315. https://doi.org/10.1016/j.jcf.2009.04.005.

Henriksen, K., Rorbo, N., Rybtke, M.L., Martinet, M.G., Tolker-Nielsen, T., Hoiby, N., Middelboe, M., Ciofu, O., Henriksen, K., Rorbo, N., Rybtke, M.L., Martinet, M.G., Tolker-Nielsen, T., Hoiby, N., Middelboe, M., Ciofu, O., 2019. *P. aeruginosa* flow-cell biofilms are enhanced by repeated phage treatments but can be eradicated by phage-ciprofloxacin combination. Pathog. Dis. 77, ftz011. https://doi.org/10.1093/femspd/ftz011.

Hentzer, M., Wu, H., Andersen, J.B., Riedel, K., B.Rasmussen, T., Bagge, N., Kumar, N., A. Schembri, M., Song, Z., Kristoffersen, P., Mane, M., W.Costerton, J., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Hùiby, N., Givskov, M., 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J. 22, 3803–3815. http://doi.org/10.1093/emboj/cdg366.

Herrmann, G., Yang, L., Wu, H., Song, Z., Wang, H., Høiby, N., Ulrich, M., Molin, S., Riethmüller, J., Döring, G., 2010. Colistin-tobramycin combinations are superior to monotherapy concerning the killing of biofilm *Pseudomonas aeruginosa*. J. Infect. Dis. 202 (10), 1585–1592. https://doi.org/10.1086/656788.

Hetrick, E.M., Shin, J.H., Paul, H.S., Schoenfisch, M.H., 2009. Anti-biofilm efficacy of nitric oxide-releasing silica nanoparticles. Biomaterials. 30 (14), 2782–2789. https://doi.org/10.1016/j.biomaterials.2009.01.052.

Ho, D.-K., Nichols, B.L.B., Edgar, K.J., Murgia, X., Loretz, B., Lehr, C.-M., 2019. Challenges and strategies in drug delivery systems for treatment of pulmonary infections. Eur. J. Pharm. Biopharm. 144, 110–124. https://doi.org/10.1016/j.ejpb.2019.09.002.

Hodson, M.E., 1995. Aerosolized dornase alfa (rhDNase) for therapy of cystic fibrosis. Am. J. Respir. Crit. Care Med. 151 (3_pt_2), S70–S74. https://doi.org/10.1164/ajrccm/151.3_Pt_2.S70.

Hoe, S., Semler, D.D., Goudie, A.D., Lynch, K.H., Matinkhoo, S., Finlay, W.H., Dennis, J. J., Vehring, R., 2013. Respirable bacteriophages for the treatment of bacterial lung infections. J. Aerosol Med. Pulm. Drug Deliv. 26 (6), 317–335. https://doi.org/10.1089/jamp.2012.1001.

Hoiby, N., 1974. *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta Pathol Microbiol Scand [B]. 82, 551–558. https://doi.org/10.1111/j.1699-0463.1974.tb02365.x.

Hoiby, N., 2011. Recent advances in the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis. BMC Med. 9, 32. https://doi.org/10.1186/1741-7015-9-32.

Hoiby, N., Bjarnsholt, T., Moser, C., Bassi, G.L., Coenye, T., Donelli, G., Hall-Stoodley, L., Hola, V., Imbert, C., Kirketerp-Moller, K., Lebeaux, D., Oliver, A., Ullmann, A.J., Williams, C., 2015. ESCMID* guideline for the diagnosis and treatment of biofilm

infections 2014. Clin. Microbiol. Infect. 21, S1–S25. https://doi.org/10.1016/j.cmi.2014.10.024.
Høiby, N., Johansen, H.K., Moser, C., Ciofu, O., Jensen, P.Ø., Kolpen, M., Mandsberg, L., Givskov, M., Molin, S., Bjarnsholt, T., 2011. *Pseudomonas aeruginosa* biofilms in the lungs of cystic fibrosis patients. Future Microbiol. 5, 167–184. https://doi.org/10.1007/978-1-4419-6084-9_10.
Howlin, R.P., Cathie, K., Hall-Stoodley, L., Cornelius, V., Duignan, C., Allan, R.N., Fernandez, B.O., Barraud, N., Bruce, K.D., Jefferies, J., Kelso, M., Kjelleberg, S., Rice, S.A., Rogers, G.B., Pink, S., Smith, C., Sukhtankar, P.S., Salib, R., Legg, J., Carroll, M., Daniels, T., Feelisch, M., Stoodley, P., Clarke, S.C., Connett, G., Faust, S. N., Webb, J.S., 2017. Low-dose nitric oxide as targeted anti-biofilm adjunctive therapy to treat chronic *Pseudomonas aeruginosa* infection in cystic fibrosis. Mol. Ther. 25 (9), 2104–2116. https://doi.org/10.1016/j.ymthe.2017.06.021.
Hunter, R.C., Asfour, F., Dingemans, J., Osuna, B.L., Hunter, R.C., Asfour, F., Dingemans, J., Osuna, B.L., Samad, T., Malfroot, A., Cornelis, P., Newman, D.K., 2013. Ferrous iron is a significant component of bioavailable iron in cystic fibrosis airways. MBio. 4, e00557–00513. https://doi.org/10.1128/mBio.00557-13.
Islan, G.A., Tornello, P.C., Abraham, G.A., Duran, N., Castro, G.R., 2016. Smart lipid nanoparticles containing levofloxacin and DNase for lung delivery. Design and characterization. Colloids Surf. B Biointerfaces. 143, 168–176. https://doi.org/10.1016/j.colsurfb.2016.03.040.
Jack, A.A., Khan, S., Powell, L.C., Pritchard, M.F., Beck, K., Sadh, H., Sutton, L., Cavaliere, A., Florance, H., Rye, P.D., Thomas, D.W., Hill, K.E., 2018. Alginate oligosaccharide-induced modification of the lasI-lasR and rhlI-rhlR quorum-sensing systems in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 62, e02318 https://doi.org/10.1128/AAC.02318-17.
Jensen, P.O., Kolpen, M., Kragh, K.N., Kuhl, M., 2017. Microenvironmental characteristics and physiology of biofilms in chronic infections of CF patients are strongly affected by the host immune response. APMIS. 125, 276–288. https://doi.org/10.1111/apm.12668.
Kenawy, E.-R., Ali, S.S., Al-Etewy, M., Sun, J., Wu, J., El-Zawawy, N., 2019. Synthesis, characterization and biomedical applications of a novel schiff base on methyl acrylate-functionalized chitosan bearing p -nitrobenzaldehyde groups. Int. J. Biol. Macromol. 122, 833–843. https://doi.org/10.1016/j.ijbiomac.2018.11.005.
Khatib, I., Tang, P., Ruan, J., Cipolla, D., Dayton, F., Blanchard, J.D., Chan, H.-K., 2020. Formation of ciprofloxacin nanocrystals within liposomes by spray drying for controlled release via inhalation. Int. J. Pharm. 578, 119045. https://doi.org/10.1016/j.ijpharm.2020.119045.
Kho, K., Cheow, W.S., Lie, R.H., Hadinoto, K., 2010. Aqueous re-dispersibility of spray-dried antibiotic-loaded polycaprolactone nanoparticle aggregates for inhaled anti-biofilm therapy. Powder Technol. 203 (3), 432–439. https://doi.org/10.1016/j.powtec.2010.06.003.
Klodzinska, S.N., Priemel, P.A., Rades, T., Morck Nielsen, H., 2016. Inhalable antimicrobials for treatment of bacterial biofilm-associated sinusitis in cystic fibrosis patients: challenges and drug delivery approaches. Int. J. Mol. Sci. 17, 1688. https://doi.org/10.3390/ijms17101688.
Knetsch, M., Koole, L.H., 2011. New strategies in the development of antimicrobial coatings: The example of increasing usage of silver and silver nanoparticles. Polymers. 3, 340–366. https://doi.org/10.3390/polym3010340.
Koehler, D.R., Downey, G.P., Sweezey, N.B., Tanswell, A.K., Hu, J., 2004. Lung inflammation as a therapeutic target in cystic fibrosis. Am. J. Respir. Cell Mol. Biol. 31 (4), 377–381. https://doi.org/10.1165/rcmb.2004-0124TR.
Kolpen, M., Mousavi, N., Sams, T., Bjarnsholt, T., Ciofu, O., Moser, C., Kühl, M., Høiby, N., Jensen, P.Ø., 2016. Reinforcement of the bactericidal effect of ciprofloxacin on *Pseudomonas aeruginosa* biofilm by hyperbaric oxygen treatment. Int. J. Antimicrob. Agents. 47 (2), 163–167. https://doi.org/10.1016/j.ijantimicag.2015.12.005.
Koo, H., Allan, R.N., Howlin, R.P., Stoodley, P., Hall-Stoodley, L., 2017. Targeting microbial biofilms: current and prospective therapeutic strategies. Nat. Rev. Microbiol. 15 (12), 740–755. https://doi.org/10.1038/nrmicro.2017.99.
Leid, J.G., Ditto, A.J., Knapp, A., Shah, P.N., Wright, B.D., Blust, R., Christensen, L., Clemons, C.B., Wilber, J.P., Young, G.W., Kang, A.G., Panzner, M.J., Cannon, C.L., Yun, Y.H., Youngs, W.J., Seckinger, N.M., Cope, E.K., 2012. In vitro antimicrobial studies of silver carbene complexes: activity of free and nanoparticle carbene formulations against clinical isolates of pathogenic bacteria. J. Antimicrob. Chemother. 67 (1), 138–148. https://doi.org/10.1093/jac/dkr408.
Li, J., Rayner, C.R., Nation, R.L., Owen, R.J., Spelman, D., Tan, K.E., Liolios, L., 2006. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother. 50 (9), 2946–2950. https://doi.org/10.1128/AAC.00103-06.
Li, Q., Zhan, S., Liu, Q., Su, H., Dai, X.i., Wang, H., Beng, H., Tan, W., 2018. Preparation of a sustained-release nebulized aerosol of r-terbutaline hydrochloride liposome and evaluation of its anti-asthmatic effects via pulmonary delivery in guinea pigs. Aaps Pharmscitech. 19 (1), 232–241. https://doi.org/10.1208/s12249-017-0816-z.
Liu, Y., Shi, L., Su, L., van der Mei, H.C., Jutte, P.C., Ren, Y., Busscher, H.J., 2019. Nanotechnology-based antimicrobials and delivery systems for biofilm-infection control. Chem. Soc. Rev. 48 (2), 428–446.
Loira-Pastoriza, C., Todoroff, J., Vanbever, R., 2014. Delivery strategies for sustained drug release in the lungs. Adv. Drug Deliv. Rev. 75, 81–91. https://doi.org/10.1016/j.addr.2014.05.017.
Manzenreiter, R., Kienberger, F., Marcos, V., Schilcher, K., Krautgartner, W.D., Obermayer, A., Huml, M., Stoiber, W., Hector, A., Griese, M., Hannig, M., Studnicka, M., Vitkov, L., Hartl, D., 2012. Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy. J. Cyst. Fibros. 11 (2), 84–92. https://doi.org/10.1016/j.jcf.2011.09.008.
Marras, T.K., Vinnard, C., Zhang, Q.W., Hamilton, K., Adjemian, J., Eagle, G., Zhang, R., Chou, E., Olivier, K.N., 2018. Relative risk of all-cause mortality in patients with nontuberculous mycobacterial lung disease in a US managed care population. Respiratory Medicine. 145, 80–88. https://doi.org/10.1016/j.rmed.2018.10.022.
Martin, D.W., Schurr, M.J., Mudd, M.H., Govan, J.R., Holloway, B.W., Deretic, V., 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. USA 90 (18), 8377–8381. https://doi.org/10.1073/pnas.90.18.8377.
Mathee, K., Ciofu, O., Sternberg, C., Lindum, P.W., Campbell, J.I.A., Jensen, P., Johnsen, A.H., Givskov, M., Ohman, D.E., Soren, M., Hoiby, N., Kharazmi, A., 1999. Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiology. 145, 1349–1357. https://doi.org/10.1099/13500872-145-6-1349.
Mauch, R.M., Jensen, P.Ø., Moser, C., Levy, C.E., Høiby, N., 2018. Mechanisms of humoral immune response against *Pseudomonas aeruginosa* biofilm infection in cystic fibrosis. J. Cyst. Fibros. 17 (2), 143–152. https://doi.org/10.1016/j.jcf.2017.08.012.
Meers, P., Neville, M., Malinin, V., Scotto, A.W., Sardaryan, G., Kurumunda, R., Mackinson, C., James, G., Fisher, S., Perkins, W.R., 2008. Biofilm penetration, triggered release and in vivo activity of inhaled liposomal amikacin in chronic *Pseudomonas aeruginosa* lung infections. J. Antimicrob. Chemother. 61, 859–868. https://doi.org/10.1093/jac/dkn059.
Merchant, Z., Buckton, G., M.G. Taylor, K., Stapleton, P., Y. Saleem, I., G. Zariwala, M., Somavarapu, S., 2016. A new era of pulmonary delivery of nano-antimicrobial therapeutics to treat chronic pulmonary infections. Curr. Pharmaceut. Des. 22 (17), 2577–2598. https://doi.org/10.2174/1381612822666160317142139.
Misagh, A., Suntres, Z.E., Abdelwahab, O., 2009a. Importance of DNase and alginate lyase for enhancing free and liposome encapsulated aminoglycoside activity against *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 64 (2), 317–325. https://doi.org/10.1093/jac/dkp165.
Misagh, A., Suntres, Z.E., Majed, H., Azghani, A.O., Abdelwahab, O., Kreindler, J.L., 2009b. Activity and interactions of liposomal antibiotics in presence of polyanions and sputum of patients with cystic fibrosis. Plos One. 4, e5724 https://doi.org/10.1371/journal.pone.0005724.
Moreau-Marquis, S., O'Toole, G.A., Stanton, B.A., 2009. Tobramycin and FDA-approved iron chelators eliminate *Pseudomonas aeruginosa* biofilms on cystic fibrosis cells. Am. J. Respir. Cell Mol. Biol. 41 (3), 305–313. https://doi.org/10.1165/rcmb.2008-0299OC.
Mugabe, C., Azghani, A., Omri, A., 2005. Liposome-mediated gentamicin delivery: development and activity against resistant strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients. J. Antimicrob. Chemother. 55, 269–271. https://doi.org/10.1093/jac/dkh518.
Muhlebach, M.S., Hatch, J.E., Einarsson, G.G., Mcgrath, S.J., Gilipin, D.F., Gillian, L., Bojana, M., Murray, M.A., Paul, M.N., Nathan, G., 2018. Anaerobic bacteria cultured from CF airways correlate to milder disease-a multisite study. Eur. Respir. J. 52, 1800242. https://doi.org/10.1183/13993003.00242-2018.
Munder, A., Tuemmler, B., 2015. Origins of cystic fibrosis lung disease. N. Engl. J. Med. 372 https://doi.org/10.1056/NEJMc1502191, 1574-1574.
Murgia, X., Pawelzyk, P., Schaefer, U.F., Wagner, C., Willenbacher, N., Lehr, C.M., 2016. Size-limited penetration of nanoparticles into porcine respiratory mucus after aerosol deposition. Biomacromolecules. 17, 1536–1152. http://doi.org/10.1021/acs.biomac.6b00164.
Nafee, N., Husari, A., Maurer, C.K., Lu, C., Rossi, C.D., Steinbach, A., Hartmann, R.W., Lehr, C.M., Schneider, M., 2014. Antibiotic-free nanotherapeutics: Ultra-small, mucus-penetrating solid lipid nanoparticles enhance the pulmonary delivery and anti-virulence efficacy of novel quorum sensing inhibitors. J. Control Release. 192, 131–140. https://doi.org/10.1016/j.jconrel.2014.06.055.
das Neves, J., Bahia, M.F., Amiji, M.M., Sarmento, B., 2011. Mucoadhesive nanomedicines: characterization and modulation of mucoadhesion at the nanoscale. Expert Opin. Drug Discov. 8 (8), 1085–1104. https://doi.org/10.1517/17425247.2011.586334.
Ng, R.N., Tai, A.S., Chang, B.J., Stick, S.M., Kicic, A., Arest, C.F., 2021. Overcoming challenges to make bacteriophage therapy standard clinical treatment practice for cystic fibrosis. Front. Microbiol. 11, 593988. https://doi.org/10.3389/fmicb.2020.593988.
Nouraldin, A.A.M., Baddour, M.M., Harfoush, R.A.H., Essa, S.A.M., 2016. Bacteriophage-antibiotic synergism to control planktonic and biofilm producing clinical isolates of Pseudomonas aeruginosa. Am. J. Med. 52 (2), 99–105. https://doi.org/10.1016/j.ajme.2015.05.002.
O'May, C.Y., Sanderson, K., Roddam, L.F., Kirov, S.M., Reid, D.W., 2009. Iron-binding compounds impair *Pseudomonas aeruginosa* biofilm formation, especially under anaerobic conditions. J. Med. Microbiol. 58, 765–773. https://doi.org/10.1099/jmm.0.004416-0.
Pamp, S.J., Gjermansen, M., Johansen, H.K., Tolker-Nielsen, T., 2008. Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Mol. Microbiol. 68 (1), 223–240. https://doi.org/10.1111/j.1365-2958.2008.06152.x.
Park, H.Y., Jeong, B.-H., Chon, H.R., Jeon, K., Daley, C.L., Koh, W.-J., 2016. Lung function decline according to clinical course in nontuberculous mycobacterial lung disease. Chest. 150 (6), 1222–1232. https://doi.org/10.1016/j.chest.2016.06.005.
Pelgrift, R.Y., Friedman, A.J., 2013. Nanotechnology as a therapeutic tool to combat microbial resistance. Adv. Drug Deliv. Rev. 65 (13-14), 1803–1815. https://doi.org/10.1016/j.addr.2013.07.011.
Powell, L.C., Pritchard, M.F., Ferguson, E.L., Powell, K.A., Patel, S.U., Rye, P.D., Sakellakou, S.-M., Buurma, N.J., Brilliant, C.D., Copping, J.M., Menzies, G.E., Lewis, P.D., Hill, K.E., Thomas, D.W., 2018. Targeted disruption of the extracellular

polymeric network of *Pseudomonas aeruginosa* biofilms by alginate oligosaccharides. NPJ Biofilms Microbiomes. 4 (1) https://doi.org/10.1038/s41522-018-0056-3.
Priemel, P.A., Wang, Y.Y., Bohr, A., Water, J.J., Yang, M.S., Nielsen, H.M., 2018. Poly (ethylene carbonate)-containing polylactic acid microparticles with rifampicin improve drug delivery to macrophages. J. Pharm. Pharmacol. 70, 1009–1021. https://doi.org/10.1111/jphp.12937.
Pritchard, M.F., Powell, L.C., Menzies, G.E., Lewis, P.D., Hawkins, K., Wright, C., Doull, I., Walsh, T.R., Onsøyen, E., Dessen, A., Myrvold, R., Rye, P.D., Myrset, A.H., Stevens, H.N.E., Hodges, L.A., MacGregor, G., Neilly, J.B., Hill, K.E., Thomas, D.W., 2015. A new class of safe oligosaccharide polymer therapy to modify the mucus barrier of chronic respiratory disease. Mol. Pharm. 13 (3), 863–872. https://doi.org/10.1021/acs.molpharmaceut.5b00794.
Pritchard, M.F., Powell, L.C., Jack, A.A., Powell, K., Beck, K., Florance, H., Forton, J., Rye, P.D., Dessen, A., Hill, K.E., Thomas, D.W., 2017. A low-molecular-weight alginate oligosaccharide disrupts Pseudomonal microcolony formation and enhances antibiotic effectiveness. Antimicrob. Agents Chemother. 61, e00762–00717. https://doi.org/10.1128/AAC.00762-17.
Qiu, D., Eisinger, V.M., Rowen, D.W., Yu, H.D., 2007. Regulated proteolysis controls mucoid conversion in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 104 (19), 8107–8112. https://doi.org/10.1073/pnas.0702660104.
Qvist, T., Eickhardt, S., Kragh, K.N., Andersen, C.B., Iversen, M., Høiby, N., Bjarnsholt, T., 2015. Chronic pulmonary disease with *Mycobacterium* abscessus complex is a biofilm infection. Eur. Respir. J. 46 (6), 1823–1826. https://doi.org/10.1183/13993003.01102-2015.
Rowe-Jones, J.M., Mackay, I.S., 1996. Endoscopic sinus surgery in the treatment of cystic fibrosis with nasal polyposis. Laryngoscope. 106 (12), 1540–1544. https://doi.org/10.1097/00005537-199612000-00019.
Roy, R., Tiwari, M., Donelli, G., Tiwari, V., 2018. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence. 9 (1), 522–554. https://doi.org/10.1080/21505594.2017.1313372.
Rumbaugh, K.P., Sauer, K., 2020. Biofilm dispersion. Nat Rev Microbiol. 18 (10), 571–586. https://doi.org/10.1038/s41579-020-0385-0.
Sabina, A., 2015. Novel inhaled combined antibiotic formulations in the treatment of *Pseudomonas aeruginosa* airways infections in cystic fibrosis. Expert. Rev. Anti. Infect. Ther. 13 (7), 897–905. https://doi.org/10.1586/14787210.2015.1041925.
Sans-Serramitjana, E., Fusté, E., Martínez-Garriga, B., Merlos, A., Pastor, M., Pedraz, J.L., Esquisabel, A., Bachiller, D., Vinuesa, T., Viñas, M., 2016. Killing effect of nanoencapsulated colistin sulfate on *Pseudomonas aeruginosa* from cystic fibrosis patients. J. Cyst. Fibros. 15 (5), 611–618. https://doi.org/10.1016/j.jcf.2015.12.005.
Schairer, D.O., Chouake, J.S., Nosanchuk, J.D., Friedman, A.J., 2012. The potential of nitric oxide releasing therapies as antimicrobial agents. Virulence. 3 (3), 271–279. https://doi.org/10.4161/viru.20328.
Schwarz, C., Grehn, C., Temming, S., Holz, F., Eschenhagen, P.N., 2021. Clinical impact of levofloxacin inhalation solution in cystic fibrosis patients in a real-world setting. J. Cyst. Fibros. 20 (6), 1035–1039. https://doi.org/10.1016/j.jcf.2021.03.002.
Scott, J.R., Krishnan, R., Rotenberg, B.W., Sowerby, L.J., 2017. The effectiveness of topical colloidal silver in recalcitrant chronic rhinosinusitis: a randomized crossover control trial. J. Otolaryngol Head Neck Surg. 46, 64. https://doi.org/10.1186/s40463-017-0241-z.
Sharma, A., Kumar, D., Dahiya, K., Hawthorne, S., Jha, S.K., Jha, N.K., Nand, P., Girgis, S., Raj, S., Srivastava, R., Goswami, V.K., Gregoriou, Y., El-Zahaby, S.A., Ojha, S., Dureja, H., Gupta, G., Singh, S., Chellappan, D.K., Dua, K., 2021. Advances in pulmonary drug delivery targeting microbial biofilms in respiratory diseases. Nanomedicine. 16 (21), 1905–1923. https://doi.org/10.2217/nnm-2021-0057.
Shetty, N., Cipolla, D., Park, H., Zhou, Q.T., 2020. Physical stability of dry powder inhaler formulations. Expert Opin. Drug Discov. 17 (1), 77–96. https://doi.org/10.1080/17425247.2020.1702643.
Simmen, H.-P., Blaser, J., 1993. Analysis of pH and pO_2 in abscesses, peritoneal fluid, and drainage fluid in the presence or absence of bacterial infection during and after abdominal surgery. Am. J. Surg. 166 (1), 24–27. https://doi.org/10.1016/S0002-9610(05)80576-8.
Singh, N., Rajwade, J., Paknikar, K.M., 2019. Transcriptome analysis of silver nanoparticles treated Staphylococcus aureus reveals potential targets for biofilm inhibition. Colloids Surf. B Biointerfaces. 175, 487–497. https://doi.org/10.1016/j.colsurfb.2018.12.032.
Singh, S., Hornick, D., Fedler, J., Launspach, J.L., Teresi, M.E., Santacroce, T.R., Cavanaugh, J.E., Horan, R., Nelson, G., Starner, T.D., Zabner, J., Durairaj, L., 2020. Randomized controlled study of aerosolized hypertonic xylitol versus hypertonic saline in hospitalized patients with pulmonary exacerbation of cystic fibrosis. J. Cyst. Fibros. 19 (1), 108–113. https://doi.org/10.1016/j.jcf.2019.06.016.
Smith, A., 2005. Biofilms and antibiotic therapy: is there a role for combating bacterial resistance by the use of novel drug delivery systems? Adv. Drug Deliv. Rev. 57 (10), 1539–1550. https://doi.org/10.1016/j.addr.2005.04.007.
Smyth, A.R., Cifelli, P.M., Ortori, C.A., Righetti, K., Lewis, S., Erskine, P., Holland, E.D., Givskov, M., Williams, P., Cámara, M., Barrett, D.A., Knox, A., 2010. Garlic as an inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis-A pilot randomized controlled trial. Pediatr. Pulmonol. 45 (4), 356–362. https://doi.org/10.1002/ppul.21193.
Solano, C., Echeverz, M., Lasa, I., 2014. Biofilm dispersion and quorum sensing. Curr. Opin. Microbiol. 18, 96–104. https://doi.org/10.1016/j.mib.2014.02.008.
Stokniene, J., Thomas, D.W., Rye, P.D., Hill, K.E., Ferguson, E.L., 2019. P103 OligoG conjugation inhibits bacterial growth and reduces cytotoxicity of colistin and polymyxin B. J. Cyst. Fibros. 18S1, S57–S190. https://doi.org/10.1016/S1569-1993(19)30397-2.
Su, S., Hassett, D.J., 2012. Anaerobic *Pseudomonas aeruginosa* and other obligately anaerobic bacterial biofilms growing in the thick airway mucus of chronically infected cystic fibrosis patients: an emerging paradigm or "Old Hat"? Expert Opin. Ther. Targets. 16 (9), 859–873. https://doi.org/10.1517/14728222.2012.708025.
Suk, J.S., Lai, S.K., Wang, Y.-Y., Ensign, L.M., Zeitlin, P.L., Boyle, M.P., Hanes, J., 2009. The penetration of fresh undiluted sputum expectorated by cystic fibrosis patients by non-adhesive polymer nanoparticles. Biomaterials. 30 (13), 2591–2597. https://doi.org/10.1016/j.biomaterials.2008.12.076.
Thomsen, K., Kobayashi, O., Kishi, K., Shirai, R., Jensen, P.O., Heydorn, A., Hentzer, M., Calum, H., Christophersen, L., Hoiby, N., Moser, C., 2021. Animal models of chronic and recurrent *Pseudomonas aeruginosa* lung infection: significance of macrolide treatment. APMIS. https://doi.org/10.1111/apm.13161.
Tian, F., Li, J., Nazir, A., Tong, Y., 2021. Bacteriophage - a promising alternative measure for bacterial biofilm control. Infect. Drug Resist. 14, 205–217. https://doi.org/10.2147/IDR.S290093.
Torge, A., Grützmacher, P., Mücklich, F., Schneider, M., 2017. The influence of mannitol on morphology and disintegration of spray-dried nano-embedded microparticles. Eur. J. Pharm. Sci. 104, 171–179. https://doi.org/10.1016/j.ejps.2017.04.003.
Torres-Barcelo, C., Hochberg, M.E., 2016. Evolutionary rationale for phages as complements of antibiotics. Trends Microbiol. 24, 249–256. https://doi.org/10.1016/j.tim.2015.12.011.
Trapnell, B.C., McColley, S.A., Kissner, D.G., Rolfe, M.W., Rosen, J.M., McKevitt, M., Moorehead, L., Montgomery, A.B., Geller, D.E., Phase, F.T.I.S.G., 2012. Fosfomycin/tobramycin for inhalation in patients with cystic fibrosis with *pseudomonas* airway infection. Am. J. Respir. Crit. Care Med. 185, 171–178. https://doi.org/10.1164/rccm.201105-0924OC.
Tseng, B.S., Zhang, W., Harrison, J.J., Quach, T.P., Song, J.L., Penterman, J., Singh, P.K., Chopp, D.L., Packman, A.I., Parsek, M.R., 2013. The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. Environ. Microbiol. 15, 2865–2878. https://doi.org/10.1111/1462-2920.12155.
Günday Türeli, N., Torge, A., Juntke, J., Schwarz, B.C., Schneider-Daum, N., Türeli, A.E., Lehr, C.-M., Schneider, M., 2017. Ciprofloxacin-loaded PLGA nanoparticles against cystic fibrosis *P. aeruginosa* lung infections. Eur. J. Pharm. Biopharm. 117, 363–371. https://doi.org/10.1016/j.ejpb.2017.04.032.
Velkov, T., Rahim, N.A., Zhou, Q., Chan, H.K., Li, J., 2015. Inhaled anti-infective chemotherapy for respiratory tract infections: Successes, challenges and the road ahead. Adv. Drug Deliv. Rev. 85, 65–82. https://doi.org/10.1016/j.addr.2014.11.004.
Wang, J., Kutter, J.P., Mu, H., Moodley, A., Yang, M., 2020a. Synergistic antibacterial effect of inhaled aztreonam and tobramycin fixed dose combination to combat multidrug-resistant Gram-negative bacteria. Int. J. Pharm. 590, 119877. https://doi.org/10.1016/j.ijpharm.2020.119877.
Wang, J., Grégoire, N., Marchand, S., Kutter, J.P., Mu, H., Moodley, A., Couet, W., Yang, M., 2020b. Improved antibacterial efficiency of inhaled thiamphenicol dry powders: Mathematical modelling of *in vitro* dissolution kinetic and *in vitro* antibacterial efficacy. Eur. J. Pharm. Sci. 152, 105435. https://doi.org/10.1016/j.ejps.2020.105435.
Wang, Q., Mi, G., Hickey, D., Li, Y., Tu, J., Webster, T.J., Shen, Y., 2018. Azithromycin-loaded respirable microparticles for targeted pulmonary delivery for the treatment of pneumonia. Biomaterials. 160, 107–123. https://doi.org/10.1016/j.biomaterials.2018.01.022.
Wang, W., Zhou, Q.T., Sun, S.-P., Denman, J.A., Gengenbach, T.R., Barraud, N., Rice, S. A., Li, J., Yang, M., Chan, H.-K., 2016. Effects of surface composition on the aerosolisation and dissolution of inhaled antibiotic combination powders consisting of colistin and rifampicin. Aaps Journal. 18 (2), 372–384. https://doi.org/10.1208/s12248-015-9848-z.
Wang, Y.u., Yuan, Q., Feng, W., Pu, W., Ding, J., Zhang, H., Li, X., Yang, B.o., Dai, Q., Cheng, L., Wang, J., Sun, F., Zhang, D., 2019. Targeted delivery of antibiotics to the infected pulmonary tissues using ROS-responsive nanoparticles. J. Nanobiotechnology. 17 (1) https://doi.org/10.1186/s12951-019-0537-4.
Weers, J., 2014. Inhaled antimicrobial therapy-Barriers to effective treatment. Adv. Drug Deliv. Rev. 85, 24–43. https://doi.org/10.1016/j.addr.2014.08.013.
Weiser, R., Rye, P.D., Mahenthiralingam, E., 2021. Implementation of microbiota analysis in clinical trials for cystic fibrosis lung infection: Experience from the OligoG phase 2b clinical trials. J. Microbiol. Methods. 181, 106133. https://doi.org/10.1016/j.mimet.2021.106133.
Wilkins, M., Hall-Stoodley, L., Allan, R.N., Faust, S.N., 2014. New approaches to the treatment of biofilm-related infections. J. Infect. 69, S47–S52. https://doi.org/10.1016/j.jinf.2014.07.014.
Wissing, S.A., Kayser, O., Müller, R.H., 2004. Solid lipid nanoparticles for parenteral drug delivery. Adv. Drug Deliv. Rev. 56 (9), 1257–1272. https://doi.org/10.1016/j.addr.2003.12.002.
Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K.C., Birrer, P., Bellon, G., Berger, J., Weiss, T., Botzenhart, K., Yankaskas, J.R., Randell, S., Boucher, R.C., Döring, G., 2002. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J. Clin. Invest. 109 (3), 317–325.
Wu, P.H., Cheng, P.C., Chang, C.M., Lo, W.C., Cheng, P.W., 2021. Efficacy of povidone-Iodine nasal irrigation solution after sinonasal surgery: A randomized controlled study. Laryngoscope. https://doi.org/10.1002/lary.29818.
Xu, J.Q., Lu, X.Y., Zhu, X.J., Yang, Y., Liu, Q., Zhao, D., Lu, Y., Wen, J.Y., Chen, X.J., Li, N., 2019. Formulation and characterization of spray-dried powders containing vincristine-liposomes for pulmonary delivery and its pharmacokinetic evaluation from *in vitro* and *in vivo*. J. Pharmaceut. Sci. 108, 3348–3358. https://doi.org/10.1016/j.xphs.2019.05.009.

Yang, M., Lai, S.K., Wang, Y.-Y., Zhong, W., Happe, C., Zhang, M., Fu, J., Hanes, J., 2011. Biodegradable nanoparticles composed entirely of safe materials that rapidly penetrate human mucus. Angew Chem. Int. Ed. Engl. 50 (11), 2597–2600. https://doi.org/10.1002/anie.201006849.

Yu, J.Y., Chuesiang, P., Shin, G.H., Park, H.J., 2021. Post-processing techniques for the improvement of liposome stability. Pharmaceutics. 13 (7), 1023. https://doi.org/10.3390/pharmaceutics13071023.

Yu, S., Yuan, H., Chai, G., Peng, K., Zou, P., Li, X., Li, J., Zhou, F., Chan, H.-K., Zhou, Q.T., 2020. Optimization of inhalable liposomal powder formulations and evaluation of their in vitro drug delivery behavior in Calu-3 human lung epithelial cells. Int. J. Pharm. 586, 119570. https://doi.org/10.1016/j.ijpharm.2020.119570.

Zhang, J., Franziska, L., Sasha, R., Yu, C.D., Jill, T., Tracey, H., Mary, N., Jayanthi, J., Perkins, W.R., Keith, D.P., 2018. Amikacin liposome inhalation suspension (ALIS) penetrates non-tuberculous mycobacterial biofilms and enhances amikacin uptake into macrophages. Front Microbiol. 9, 915-. https://doi.org/10.3389/fmicb.2018.00915.

Zhang, Q., Zhang, F., Chen, Y., Dou, Y., Tao, H., Zhang, D., Wang, R., Li, X., Zhang, J., 2017. Structure–property correlations of reactive oxygen species-responsive and hydrogen peroxide-eliminating materials with anti-oxidant and anti-inflammatory activities. Chem. Mater. 29 (19), 8221–8238. https://doi.org/10.1021/acs.chemmater.7b0241210.1021/acs.chemmater.7b02412.s001.

Zhang, Y., Hill, A.T., 2021. Amikacin liposome inhalation suspension as a treatment for patients with refractory *mycobacterium avium* complex lung infection. Expert Rev. Respir. Med. 15 (6), 737–744. https://doi.org/10.1080/17476348.2021.1875821.