Lipid and polymer nanoparticles for drug delivery to bacterial biofilms

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Keywords

biofilm; nanoparticle; drug delivery; nanomedicines; liposome; polymer nanoparticles; lipid polymer hybrid nanoparticles; antibiotics; diffusion

Abstract

Biofilms are matrix-enclosed communities of bacteria that show increased antibiotic resistance and the capability to evade the immune system. They can cause recalcitrant infections which cannot be cured with classical antibiotic therapy. Drug delivery by lipid or polymer nanoparticles is considered a promising strategy for overcoming biofilm resistance. These particles are able to improve the delivery of antibiotics to the bacterial cells, thereby increasing the efficacy of the treatment. In this review we give an overview of the types of polymer and lipid nanoparticles that have been developed for this purpose. The antimicrobial activity of nanoparticle encapsulated antibiotics compared to the activity of the free antibiotic is discussed in detail. In addition, targeting and triggered drug release strategies to further improve the antimicrobial activity are reviewed. Finally, ample attention is given to advanced microscopy methods that shed light on the behavior of nanoparticles inside biofilms, allowing further optimization of the nanoformulations. Lipid and polymer nanoparticles were found to increase the antimicrobial efficacy in many cases. Strategies such as the use of fusogenic liposomes, targeting of the nanoparticles and triggered release of the antimicrobial agent ensured the delivery of the antimicrobial agent in close proximity of the bacterial cells, maximizing the exposure of the biofilm to the antimicrobial agent. The majority of the discussed papers still present data on the *in vitro* anti-biofilm activity of nanoformulations, indicating that there is an urgent need for more *in vivo* studies in this field.

1. Introduction

In the late 1970's, the biofilm mode of growth was recognized as the predominant form in which bacteria are present in many different environments. [1] Although seemingly trivial at that time, this discovery has had a profound impact on our understanding of the pathogenesis and treatment of bacterial infections. [2-5] Today, treating biofilm infections is one of the major challenges the medical community is facing and is expected to remain so for many years to come.

By definition, biofilms are matrix-enclosed, complex and differentiated communities of bacteria that are adherent to inert or biological surfaces. [6] Upon adhesion, the bacterial cells start producing extracellular matrix and group together in densely packed bacterial clusters. From the mature biofilm, individual cells or biofilm fragments are released and can colonize new surfaces (Figure 1). [7] It was observed that biofilm associated bacteria, termed sessile cells, display a profoundly different phenotype compared to their free swimming, planktonic counterparts. [7] The biofilm bacteria are able to communicate and alter each other's phenotype by a process called quorum sensing. [8-10] This allows the biofilm to respond cooperatively to environmental changes and threats. Thus, the biofilm mode of growth is an adaptation which allows the bacteria to survive and thrive in otherwise hostile environments.

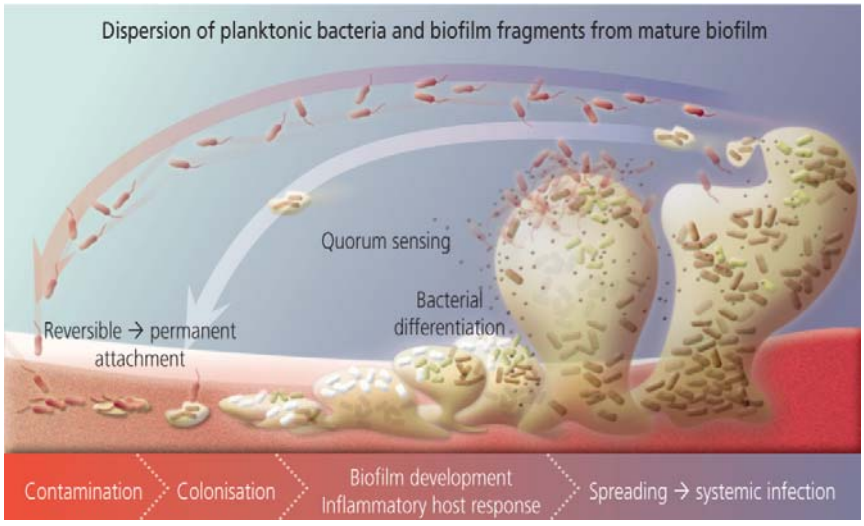

Figure 1: Biofilm formation and dispersal. Reprinted with permission from [11].

Besides their presence in natural and industrial settings, biofilms can also form in the human body, causing recalcitrant infections. [12] Parsek and Singh have proposed a set of criteria to determine if biofilm formation is involved in an infection. [5] Firstly, the bacteria must be surface associated. Secondly, the bacteria must be present in clusters or microcolonies embedded in an extracellular matrix. Thirdly, the infection should be confined to a particular location and finally, the infection cannot be eradicated using classic antibiotic therapy. The reason why these infections are hard to eradicate is twofold. On the one hand, the biofilm bacteria display increased antimicrobial resistance and tolerance compared to planktonic

bacteria. [13-15] On the other hand, the biofilm mode of growth enables the bacteria to evade the immune system of the host. [16, 17] As a consequence, biofilms can cause devastating chronic infections and are associated with an increased morbidity and mortality. It is now estimated that over 60% of bacterial infections in humans involve biofilm formation. [17] As a consequence, the economic burden due to biofilm infections is substantial. For example, catheter related sepsis costs an additional $ 28,000 per case. Nosocomial urinary tract infections, which are a subset of these catheter related infections, account for approximately 900,000 admissions annually in the US. [18]

The biggest challenge in treating biofilm infections is overcoming the resistance and tolerance against antimicrobial agents. Several mechanisms of antimicrobial resistance and tolerance have been suggested such as limited diffusion of antimicrobial agents in the biofilm matrix, deactivation of the antimicrobial agent in the outer layers of the biofilm via binding to matrix components or enzymatic modification and the occurrence of niches in the biofilm with less sensitive cells, including starved cells and persister cells. [15, 17, 19-21] There is an urgent need for innovative strategies that are able to overcome these mechanisms of resistance. One possible approach which is gaining considerable interest is the use of nanoparticles for antimicrobial drug delivery.

The number of publications involving the use of nanomedicines in the prevention of biofilm formation and the eradication of existing biofilms has been growing steadily over the past decade, with special attention going to lipid and polymer nanoparticles. Attractive properties of these particles are their biocompatibility, the versatility of materials and surface modifications, the possibility for targeting and triggered release, their ability to incorporate lipophilic as well as hydrophilic drugs and a reduction of unwanted side effects of the drug. [22, 23] In the context of treating biofilm infections, the use of nanoparticles to encapsulate antimicrobial agents might have several benefits. The nanoparticles can protect the antimicrobial agent from binding to matrix material and enzymatic inactivation. Lipid nanoparticles can fuse with the bacterial outer membrane, delivering the antimicrobial agent directly to the bacterial cells. Furthermore, by targeting of the nanoparticles to the biofilm, a high dose of antimicrobial agents can be delivered in the direct proximity of the bacterial cells, thereby maximizing therapeutic benefit while reducing unwanted side effects.

In this review, an overview of lipid and polymer nanoparticles for drug delivery to bacterial biofilms is provided. First, the delivery of antimicrobial agents to bacterial biofilms is discussed. A distinction is made between particles used for non-targeted delivery, particles for targeted delivery and particles that are designed to release the antimicrobial agent upon application of an external trigger. Next, methods that allow quantification of transport and interactions of nanoparticles in bacterial biofilms are discussed as they can provide essential information to optimize the nanoparticle formulations. Table 1 lists all the liposomal formulations that were tested in the context of drug delivery to biofilms and Table 2 all the polymer and lipid-polymer hybrid nanoparticles. For the abbreviations of the lipids and polymers used we refer to the legends of Table 1 and 2.

2. Non-targeted delivery to biofilms

2.1 Lipid nanoparticles

Due to their versatility and biocompatibility, liposomes are attractive candidates for nanoparticle mediated drug delivery in biofilms. Liposomes are spherical vesicles consisting of one or more phospholipid double layers. Lipophilic drugs can be incorporated into the phospholipid double layers while hydrophilic drugs can be encapsulated into the aqueous core. Several authors have reported that the encapsulation of antibiotics in liposomes resulted in lower minimal inhibitory concentrations (MIC) for clinically relevant biofilm forming organisms and/or lower minimal biofilm inhibitory concentrations (MBIC) compared to the free antibiotic *in vitro*. Different mechanisms for this increased activity of nanoparticle encapsulated antimicrobial agents have been proposed. Similarly, micelles, which consist of a single phospholipid layer, can also be used as drug delivery vehicles. In contrast to liposomes, these particles can only carry lipophilic drugs.

2.1.1 Fusogenic liposomes

One of the biggest advantages of using liposomes as drug delivery vehicles, is their potential to fuse with phospholipid membranes and deliver the antibiotic directly to the cells. Fusogenic liposomes consist of lipids that make the lipid bilayer more fluid and can promote destabilization of biological membranes. Liposomes containing a phosphoethanolamine (PE) moiety such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) are well-known examples. [24-26] The fluidity of liposomes can be tuned by the choice of phospholipids. In general, the shorter the acyl chains of the phospholipids, the lower the phase transition temperature (Tc) of the liposomes will be. The presence of double bounds or asymmetry in the acyl chains tends to lower the Tc of the liposomes as well since this induces a disturbance in the packing of the phospholipid bilayer. [27] Also the addition of cholesterol lowers the Tc. [28]

It was found that the bactericidal activity of liposomal tobramycin primarily depends on the fluidity of the liposomal membrane. DPPC:DMPG liposomes, called Fluidosomes™, with a Tc of approximately 30 °C showed a significant decrease in viability of *Pseudomonas aeruginosa* biofilms compared to the free antibiotic. *P. aeruginosa* is known for causing chronic pulmonary infections in cystic fibrosis (CF) patients and patients suffering of non-CF bronchiectasis, but can also cause wound and device related infections. Two very recent reviews on the use of lipid, polymer and lipid polymer hybrid nanoparticles for the treatment of chronic *P. aeruginosa* lung infections have been published. [29, 30] Using Fluidosomes™, complete eradication of a chronic pulmonary *P. aeruginosa* infection was obtained in an *in vivo* rat model. The antibiotic was found to remain manly in the lungs after liposomal delivery compared to the free drug, which likely reduces systemic side effects and toxicity. However, no increased antimicrobial effect of DSPC:DMPC liposomes with a Tc around 44 °C was observed. [31] The antimicrobial activity of the Fluidosomes™ was further compared to that of free tobramycin in *in vitro* biofilms of *P. aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia*, which are CF pathogens, and *Escherichia coli* and *Staphylococcus aureus.* Fluidosomes™ showed an increased antimicrobial effect compared to the free antibiotic in all biofilms tested, meaning that the

advantage of Fluidosomes™ is not restricted to one species of bacteria. [32, 33] Experimental evidence for membrane fusion of Fluidosomes™ was obtained from flow cytometry and from lipid mixing studies that both showed mixing of the lipids from the Fluidosomes™ with the membrane of the bacteria (Figure 2 A & B). For flow cytometry, Fluidosomes™ were labeled with a membrane-inserting fluorescent probe. After incubation and removal of non-adsorbed liposomes, an increase in fluorescence of the bacteria was measured indicating incorporation of the lipids from the Fluidosomes™ into the bacterial outer membrane. For the lipid-mixing assay, two membrane inserting fluorescent probes that show fluorescence resonance energy transfer (FRET) were used to label the Fluidosomes™. The change in FRET efficiency when the Fluidosomes™ were incubated with the bacteria was indicative of a fusion process. Electron microscopy on the other hand showed that immunogold labeled antibiotics could be found internalized in the bacterial cells to a higher degree when delivered by Fluidosomes™ as compared to the free antibiotic (Figure 2 D & E). In addition, the electron microscopy images showed close contact between the liposomes and the outer membrane of the bacteria (Figure 2 C). [34] Surprisingly, when the Fluidosomes™ were loaded with meropenem, higher MIC values compared to the free antibiotic were obtained in *P. aeruginosa* biofilms. [35] The explanation for this difference with tobramycin is likely an interaction between meropenem and the liposomal membrane that inhibits the fusion between bacteria and the liposomes. Fluidosomes™-tobramycin are currently further developed by Axentis Pharma (Zurich, Switzerland) for the treatment of *B. cepacia* infections in CF patients. No further information about ongoing clinical trials with this formulation is available.

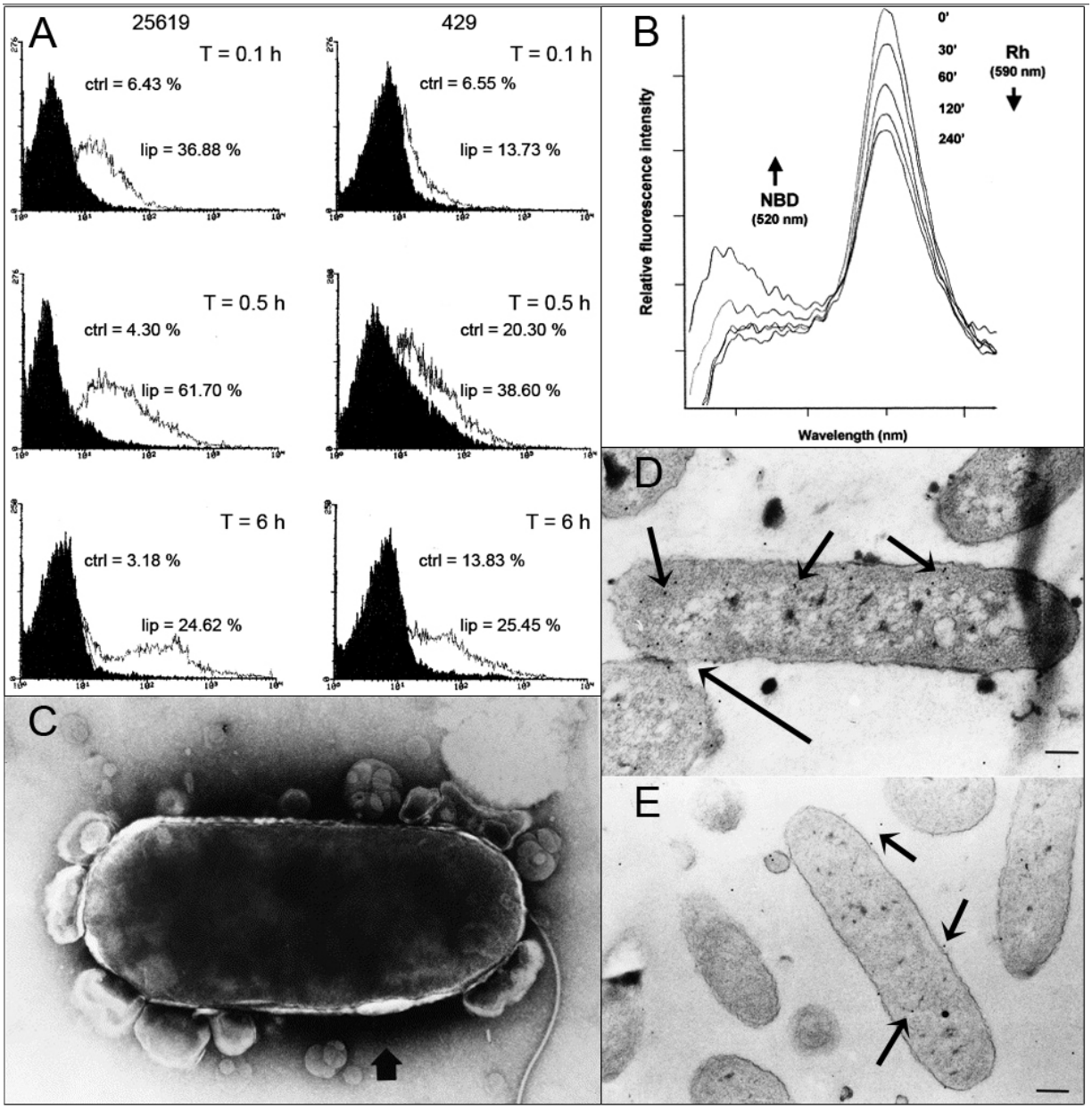

Figure 2: (A) Flow cytometry histograms of two P. aeruginosa strains (25619 and 429) incubated with free fluorescent dye (ctrl, filled graphs) or with fluorescently labeled Fluidosomes™ (lip, open graphs). The percentage of fluorescent cells is higher for the liposome treated bacteria. (B) Lipid mixing assay using rhodamine (Rh) and N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD) as fluorescent dyes. The relative change in the fluorescence intensities of both dyes indicate a change in the distance between both dyes. (C) Interaction of Fluidosomes™ with the outer membrane of a P. aeruginosa cell. The arrow shows thickening of the outer membrane, likely as a consequence of fusion of the liposomes with the membrane. Magnification: x50561. (D) Immunogold staining of tobramycin delivered by Fluidosomes™ or in the free form (E). Regions with gold nanoparticles (dense black spheres) are indicated with arrows. Magnification (D): x41126, (E): x36720. Reprinted with permission from [34]

Increased antimicrobial activity of amikacin, gentamicin and tobramycin containing liposomes either composed of DPPC:Chol or DSPC:Chol was observed in *P. aeruginosa* or *Burkholderia cenocepacia* biofilms respectively. Fusion of these liposomes with the bacteria was also proven by a combination of flow cytometry, lipid mixing, TEM and immunochemistry techniques as mentioned above. [36, 37] Of interest is a comparative study between free vancomycin and two types of liposomal formulations. Contradictory to the previously mentioned study, DPPC:Chol liposomes were regarded as classic non-fusogenic liposomes and as fusogenic liposomes DPPC:DOPE:CHEMS liposomes were used. Activity tests were performed on the gram negative bacteria *E. coli*, *Klebsiella* spp., *P. aeruginosa* and *Acinetobacter baumannii*, which are opportunistic bacteria that are intrinsically resistant to vancomycin since its high molecular weight prevents it from passing through the outer membrane. As expected, free vancomycin and the non-fusogenic liposomal formulation had no activity against the tested strains, while the fusogenic liposomes did. Electron microscopy images confirmed the interaction and possible fusion of the fusogenic liposomes with the outer membrane of *E. coli* cells. [25]

The fluidity of the liposomal membrane also has an influence on the transport of the liposomal formulation to the site of infection. For example, flexible daptomycin-containing liposomes composed of soy phosphatidylcholine and sodium cholate were found to rapidly distribute in skin tissue, making them suitable for anti-biofilm topical skin therapy. After topical application they were found to inhibit *S. aureus* biofilm growth onto subcutaneously implanted silicone membranes in mice to the same extent as intravenous injection of daptomycin. [38]

In addition to the specific liposomal composition, the properties of the bacterial outer membrane might influence fusogenicity as well. The latter was extensively investigated by Drulis-Kawa and colleagues for liposome interaction with *P. aeruginosa* cells. The outer membrane proteins, lipopolysaccharides (LPS), hydrophobicity and electrostatic potential of the bacterial cells were taken into account. [39] Using PC:Chol:DOTAP liposomes containing the fluorophore rhodamine B, no influence of outer membrane proteins involved in the efflux pump system on the fusion was found. Likewise, the LPS did not have a significant impact on the interactions with the liposomes since strains exposing the same kind of LPS showed a different uptake of liposomal rhodamine B. The authors conclude that mainly hydrophobic and electrostatic interactions play a role in the initial binding of the liposomes but that other elements or structures are likely involved in strengthening the interaction. An 18 kDa outer membrane protein was observed in *P. aeruginosa* strains that showed uptake of liposomal rhodamine B, suggesting that this protein might have a role in the fusion of the liposomes with the outer membrane. [39]

2.1.2 Protection of the antimicrobial agent

Nanoparticles can shield their cargo from the surrounding medium and protect it from enzymatic inactivation or binding to the biofilm matrix or other components surrounding the biofilm infection site. The degradation of β-lactam antibiotics by β-lactamases, the binding of aminoglycoside antibiotics to the alginate matrix of mucoid *P. aeruginosa* biofilms and the inhibition of the activity of tobramycin by lung mucus in cystic fibrosis patients are all examples of unwanted interactions of the antimicrobial agent with the biofilm or the surrounding tissue. [20, 40, 41] Mugabe et al. evaluated the antimicrobial activity of three different liposomes

(DMPC:Chol, DPPC:Chol, DSPC:Chol) encapsulating gentamycin against biofilms of several clinical strains of *P. aeruginosa*. [42] All liposomes showed a significantly higher antimicrobial activity compared to the free antibiotic. The authors attribute this effect to protection of gentamycin against binding or enzymatic inactivation. An example of protection of the β-lactam antibiotic piperacillin against staphylococcal β-lactamase by encapsulation into PC:Chol liposomes was given by Nacucchio et al. Encapsulated piperacillin showed a higher activity against *S. aureus* compared to the free piperacillin, even when exogenous β-lactamase was added. [43] In cystic fibrosis (CF) sputum, tobramycin or polymyxin B incorporated in DMPC:Chol or DPPC:Chol liposomes, respectively, were shown to be protected from binding to polyanionic polymers commonly found in CF mucus. The liposome encapsulated antibiotics demonstrated a significant increase in antimicrobial activity towards *P. aeruginosa* bacteria in the presence of DNA, F-actin, lipopolysaccharides and lipoteichoic acid. [44]

It is worth noting that co-encapsulation of other antimicrobial substances together with an antibiotic into liposomes could improve the antimicrobial efficacy. Omri and coworkers showed that the co-encapsulation of gallium or bismuth-ethanedithiol with gentamycin or tobramycin in DPPC:Chol or DSPC:Chol, respectively, can increase the vulnerability of the bacterial cells and interfere with the expression of virulence factors, alginate, quorum sensing molecules and biofilm formation compared to the free antibiotic or the free antibiotic and the free metal combined. The cytotoxicity of the gallium or bismuth-ethanedithiol containing particles was evaluated on a human lung carcinoma epithelial cell line. The toxicity was reduced for the liposomal metals compared to the free metals. [45, 46] Co-encapsulation thus seems to facilitate a synergistic effect between the antibiotic and the metals. [45-50]

2.1.3 Prolonged contact time: treatment and prevention of biofilm infections

Also, longer contact times between the antibiotic and the biofilm bacteria have been suggested to be responsible for the increased antimicrobial effect. [51, 52] This can for example be achieved by using liposomes that have a surface charge opposite to the surface charge of the bacteria. The antimicrobial activity of clarithromycin encapsulated into negatively (DPPC:DCP:Chol), positively (DPPC:DDAB:Chol) or neutrally charged (DPPC:Chol) liposomes was tested against 9 *P. aeruginosa* strains. [52] With the negatively and positively charged liposomal formulation, complete eradication of the biofilms could be achieved. The positively charged liposomes were able to eradicate the biofilms at a lower concentration than the negatively charged liposomes. This could be attributed to greater binding of the positively charged liposomes to the negative bacteria due to electrostatic attraction, possibly inducing fusion with the outer membrane. The cytotoxicity of these liposome preparations and the free drug was investigated on a human lung carcinoma epithelial cell line. Clarithromycin incorporated into the neutral or negatively charged liposomes was found to be significantly less cytotoxic than clarithromycin incorporated into positively charged liposomes and the free clarithromycin.

Longer contact times between the biofilm and the antimicrobial agent can also be obtained by incorporating liposomes into biodegradable scaffolds. These have mainly been designed and tested in the context of chronic osteomyelitis, where the skin associated *S. aureus* is the main pathogen. A porous β-tricalciumphosphate biodegradable scaffold impregnated with gentamycin sulfate encapsulated into DPPC:Chol or a nano-hydroxyapatite/β-

tricalciumphosphate scaffold impregnated with PC:SA:Chol liposomes containing ceftazidime showed increased anti-biofilm activity against *S. aureus* compared to the free drug. Both formulations showed an initial burst release followed by a slower, sustained release of the antibiotic. [53, 54] With a nano-hydroxyapatite/chitosan/konjac glucomannan scaffold containing liposomal (PC:SA:Chol) vancomycin, better inhibition of *S. aureus* biofilm growth was observed compared to the scaffold containing the free drug or the scaffold alone. This was attributed to sustained release of vancomycin from the liposomes. [55]

On the other hand, liposomes can also be used for the prevention of colonization and subsequent biofilm formation on a surface. For the prevention of orthopedic device related osteomyelitis, a biomineral-binding liposomal formulation which could be loaded with different antibiotics was developed. To achieve binding to hydroxyapatite, an alendronate-tri(ethyleneglycol)-cholesterol conjugate was designed and used together with DSPC to form liposomes for the encapsulation of oxacillin. The potential of these 'biomineral binding liposomes' (BBL) to inhibit *S. aureus* biofilm growth on hydroxyapatite discs pretreated with this formulation was investigated. The BBL showed strong affinity and rapid binding to hydroxyapatite. This allows to quickly load the liposomes onto the implant surface just before surgery. Furthermore, a fast release of most of the oxacillin from the BBL during 6 hours was observed, which can reduce the likelihood of surgery-related bacterial colonization and subsequent chronic prosthetic joint infection. The BBL pretreated discs showed almost complete biofilm inhibition. [56] Similarly, liposomes that can adhere to the dental surface can prevent caries by sustained release of antimicrobial agents. It was shown that cariogenic *Streptococci* can deposit insoluble glucans on the dental enamel which provide support for bacterial colonization. The encapsulation of nisin, which is able to inhibit the glucan synthesis by *S. mutans*, into liposomes composed of PC:PS prolonged the period over which the glucan synthesis was inhibited due to controlled release from the liposomes. [57] It should be mentioned that micelles, which in contrast to liposomes consist only of a single layer of amphiphilic molecules, were proposed for the prevention of dental caries for the same reasons as liposomes. Micelles of Pluronic 123-alendronate and Pluronic P85 containing the hydrophobic antibacterial agent farnesol were formed. The alendronate moiety has a high affinity for hydroxyapatite and caused the micelles to quickly bind to the hydroxyapatite. The tested tooth binding micelles were capable of preventing *S. mutans* biofilm formation on hydroxyapatite discs, even after extensive washing. [58] The prevention of catheter related infections on the other hand can be achieved by embedding liposomal ciprofloxacin into a gelatin-polyethylene glycol (PEG) hydrogel that can be applied to silicone catheter material. Ciprofloxacin was released from this system for seven days. When challenging the system with *P. aeruginosa*, adhesion of the bacteria was completely inhibited. [59]

2.1.4 In vivo studies

A few studies made the transition from *in vitro* to *in vivo* evaluation and used a rat model for chronic pulmonary infections with *P. aeruginosa* to compare classical treatment with the liposomal formulation. [31, 51, 60] Meers et al encapsulated amikacin into DPPC:Chol liposomes. In an *in vivo* model for chronic lung infections, rats infected with *P. aeruginosa* were treated using nebulized liposomal or free amikacin. [51] The free amikacin was relatively ineffective while the liposomal amikacin was able to reduce the amount of colony forming units

by two orders of magnitude. The liposomal formulation was found to maintain higher antibiotic levels in the lungs over longer periods of time than the free antibiotic, explaining the improved efficacy. In one study however, the liposomal formulation of antibiotics did not achieve the desired effect. [60] The activity of tobramycin encapsulated in DPPC:DMPG liposomes was evaluated *in vivo* against *B. cepacia*. Encapsulation in liposomes lead to slower clearance of tobramycin from the lungs, increasing the level of exposure of the bacteria to the drug. Twelve hours after intratracheal administration of the free or liposomal drug, a significant difference could be noted in the amount of colony forming units cultured from the lungs of the rats while this was not the case in the first 12 hours. This proves the beneficial effect of a prolonged exposure. Although encapsulation into liposomes improved the efficacy of the treatment, a higher antimicrobial effect than currently observed was expected for the liposomes since the amount of tobramycin detected in the lungs was much higher for the liposomal formulation compared to the free tobramycin. The authors attribute this to the difference in localization of the liposomes and the infection site in the lungs. The liposomes are likely sequestered in more lipophilic environments of the lungs while the bacteria are more likely to reside in the interstitial fluids. Thus, the total amount of antibiotic recovered from the lungs in the experiment is not necessarily representative for the amount at the infection site.

In vivo tests with liposomes were also conducted in the context of prevention and treatment of osteomyelitis. The treatment of osteomyelitis with liposomal (DPPC:SA:Chol) gentamycin impregnated calcium sulfate in a rabbit model yielded promising results over treatment with gentamycin impregnated calcium sulfate or liposomal gentamycin alone. When using the liposomal formulation, gentamycin was released for at least 12 days with a burst release directly after implantation. Complete sterilization of the bones was only observed in cultures taken from animals treated with the liposomal gentamycin impregnated calcium sulfate. [61]

A number of companies are developing liposomal formulations for the treatment of biofilm infections which are currently being evaluated in clinical trials. For the treatment of *Candida albicans*, a pathogen that is able to form fungal biofilms and cause infections in immunocompromised patients, several liposomal formulations, AmBisome® (Astellas, Northbrook, IL, USA), Abelcet® (Sigma-Tau PharmaSource, Inc., Indianapolis, IN, USA) and Amphotec® (Ben Venue Laboratories, Inc., Bedford, OH, USA), are already on the market. There are currently no liposomal formulations on the market for the treatment of bacterial biofilm infections. Arikace®, which is based on the above mentioned publication by Meers et al., is to the best of our knowledge the only nanoformulation for the treatment of bacterial biofilm infections that is currently in phase III clinical trials. The results of the phase II clinical trial have been published and showed a durable improvement in lung function and a reduced *P. aeruginosa* sputum count. [62] Furthermore, Arikace® is also being investigated as a treatment for nontuberculous mycobacterial lung disease (phase II clinical trial) and for non-CF bronchiectasis patients with *P. aeruginosa* lung infections (phase II clinical trial), which both likely involve biofilm formation. No clinical data are available at this moment. Pulmaquin™ and Lipoquin™ (Aradigm, Hayward, CA, USA) are liposomal formulations of ciprofloxacin and have both been tested in a phase IIb clinical trial for non-CF bronchiectasis involving chronic *P. aeruginosa* infections. The two formulations differ in release kinetics, with Pulmaquin™ showing the slowest release. Lipoquin™ was also evaluated for the treatment of pulmonary infections in

cystic fibrosis patients in a phase IIa clinical trial. [63] Fluidosomes™-tobramycin (Axentis Pharma, Zurich, Switzerland) is another liposomal formulation for the treatment of bacterial lung infections in CF patients. In this case, *Burkholderia cepacia* bacteria are the prime target. However, no information on the progress regarding Fluidosomes™-tobramycin in clinical trials is available at the moment. MiKasome (NeXstar Pharmaceuticals, Inc., Boulder, COL, USA), is a liposomal amikacin formulation for complicated urinary tract infections, whose development was stopped after a phase II clinical trial.

2.1.5 Absence of increased anti-biofilm effect

Not in all studies an increased anti-biofilm effect was observed for liposome encapsulated antimicrobial agents. No increased anti-biofilm effect was observed for tobramycin encapsulated in neutral DPPC:Chol or in anionic DOPC:DPPG liposomes in 5 different *B. cepacia* complex strains. For the anionic liposomes, electrostatic repulsion could limit fusion events. Fusion of the neutral liposomes with the bacteria could be hampered by a layer of matrix polymers in close proximity to the bacteria. The relatively large size of the neutral liposomes, i.e. 430 nm, could also limit their penetration into the biofilm, limiting the available amount of antibiotic in the denser parts of the biofilm. [64] When comparing the activity of two types of cationic liposomes, DOTAP:DOPE:PC or DOTAP:Chol:PC, loaded with either meropenem, gentamycin or ciprofloxacin on nine different ATCC strains of *P. aeruginosa*, *Klebsiella pneumoniae* and *E. coli*, variable results were obtained when comparing the MIC values of the liposomal and free form of the drugs. [65]. Most of the tested strains were more sensitive towards both liposomal formulations of ciprofloxacin while a lower number of strains were more sensitive to liposomal meropenem and almost no strains were more sensitive to liposomal gentamicin. For ciprofloxacin and gentamycin, the electrostatic interaction between the liposomes and the bacteria can increase the amount of antibiotic transported through the outer membrane. The slight amphiphilic character of meropenem causes it to pass the inner membrane easily while gentamycin can pass the inner membrane by means of hydrophobicity. [66] The increased intracellular concentration of the drug leads to higher antibacterial efficacy of the liposomal formulation. For gentamycin, ionic binding to the outer membrane of the bacteria is important for its antimicrobial activity. [67] When encapsulated into liposomes, this binding of the drug to the outer membrane is hampered, explaining the lack of efficacy of liposomal gentamycin towards all the tested strains. [65] The physicochemical properties of the antibiotics and the location of their target in the bacterial cells can thus influence the antimicrobial activity of the liposomal formulation.

In addition to its detrimental effect on the free antibiotic, the environment in which the biofilm is residing can also influence the antimicrobial efficacy of liposomal antimicrobial agents. For example, when treating pulmonary infections in cystic fibrosis patients, mucin, DNA and F-actin present in the lung mucus and alginate produced by mucoid *P. aeruginosa* strains might prevent liposome-bacteria interactions. In this case, no advantage of the liposomal (DMPC:Chol) tobramycin, gentamycin and amikacin formulations over the free drug was observed. This can be attributed to the increase in viscoelasticity which limits the diffusion of the liposomes or a stabilization of the liposomes by the mucus which reduces the release of the antibiotic from the liposomes. [44] Alternatively, the liposomes might bind to alginate, preventing the nanocarrier to bind to the bacteria themselves or destabilizing the liposomes leading to premature release

and subsequent inactivation of the antibiotic. [68] Although the addition of DNase and alginate lyase improved the activity of the free and liposomal antibiotics, no superiority of the liposomal formulation was observed. Likely, alginate-derived oligosaccharides still prevents the interaction of the liposomes with the bacterial membrane or still causes a destabilization of the liposomes. Nonetheless, this approach presents a viable way of enhancing the antimicrobial activity and can possibly be improved and extended to other biofilms.

2.1.6 Encapsulation efficiency

In order to deliver a sufficiently high dose of antimicrobial agents to the biofilm, a high encapsulation efficiency of the antimicrobial agent in the nanocarrier is required. In the case of liposomes, some options to improve the encapsulation efficiency are available. The options include active drug loading, reverse phase evaporation and dehydration-rehydration (freeze-drying). We refer the reader to a recent review where this was described in detail. [69] Of note is an optimized encapsulation method by Mugabe et al. which made use of freeze-drying of the liposomes in the presence of sucrose to improve the loading of aminoglycoside and macrolide antibiotics into DPPC:Chol liposomes. [70] These two classes of antibiotics are known for their low encapsulation efficiency into liposomes. By using this optimized method, the encapsulation efficiency is now high enough to use them for liposomal drug delivery. This optimized protocol was used to prepare the liposomes in several studies discussed in this review. [36, 37, 44, 68]

Table 1: Liposomes tested for drug delivery to biofilms. Used abbreviations: ALN-TEG-Chol: 8-(cholest-5-en-3β-xyloxy)-3,6-dioxaoctanyl alendronate, CHEMS: Cholesterol hemisuccinate, Chol: Cholesterol, Con-A: Concanavalin A, DC-Chol: 3-β[N(N1N1-dimethylaminoethane-carbamoyl] cholesterol, DCP: Dicetyl phosphate, DDAB: Dimethyldioctadecylammoniumbromide, DMPC: 1,2-dimyristoyl-sn-glycero-3-phosphocholine, DMPG: 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol), DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane, DPPA: 1,2-Dipalmitoyl-sn-glycero-3-phosphate, DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPG: 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol), DPTAP: Dipalmitoyl trimethylammoniumpropane, DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine, DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, ND: Not determined, PC: Phosphatidylcholine, PEG: Poly(ethyleneglycol), PI: Phosphatidylinositol, PS: Phytosphingosine, SA: Stearylamine, WGA: Wheat germ agglutinin

Nanoparticle composition	Antimicrobial agent	Size (µm)	Charge	Interaction with the bacterial membrane ?	Triggered release?	Improved antimicrobial effect?	Mechanism of action	Biofilm	Reference
DPPC:DMPG	Tobramycin	0.4	Anionic	Yes	ND	Yes	Fusion with bacterial membrane	*P. aeruginosa* *S. maltophilia* *B. cepacia* *E. coli*	[32-34]

								S.aureus	
DPPC:Chol	Amikacin Gentamicin Tobramycin	0.2	Neutral	Yes	ND	Yes, complete eradication	Fusion with bacterial membrane	*P. aeruginosa*	[37]
DSPC:Chol	Amikacin Gentamicin Tobramycin	0.2	Neutral	Yes	ND	Yes	Fusion with bacterial membrane	*B. cenocepacia*	[36]
DPPC:Chol DPPC:DOPE:CHEMS	Vancomycin	0.1	Neutral	Yes	ND	No Yes	Fusion with bacterial membrane	*E. coli* *P. aeruginosa* *A. baumannii* *Klebsiella* spp.	[25]
DPPC:DMPG DSPC:DMPC	Tobramycin	0.4	Anionic Neutral	Yes Yes	ND	Yes, complete eradication No	Fusion with bacterial membrane (*in vivo* study)	*P. aeruginosa*	[31]
DPPC:DMPG	Meropenem	0.12	Neutral	No	ND	No	Inhibition of fusion with bacterial membrane	*P. aeruginosa*	[35]
Soy-PC:Sodium cholate	Daptomycin	0.055	Neutral	ND	ND	ND	Increased penetration of daptomycin into the biofilm (*in vivo* study)	*S. aureus*	[38]
DMPC:Chol DPPC:Chol DSPC:Chol	Gentamycin	0.4	Neutral	ND	ND	Yes	Protection of gentamycin against degradation or fusion with bacterial membrane	*P. aeruginosa*	[42]
PC:Chol	Piperacillin	ND	Neutral	ND	ND	Yes	Protection against β-lactamase degradation	*S. aureus*	[43]
DMPC:Chol DPPC:Chol	Tobramycin Polymyxin B	0.3 0.45	Neutral	ND	ND	Yes Yes	Protection against binding to non-target materials and degradation	*P. aeruginosa*	[44]
DPPC:Chol DSPC:Chol	Gentamycin-Gallium Tobramycin-Bismuth	0.34 0.9	Neutral Neutral	ND ND	ND ND	Yes Yes, complete eradication	Increased vulnerability of the bacteria against co-encapsulated metal, interference with quorum sensing, virulence	*P. aeruginosa*	[45-50]

							factor production and biofilm formation		
DDAB:DPPC:Chol DCP:DPPC:Chol DPPC:Chol	Clarithromycin	0.17 0.2 0.22	Cationic Anionic Neutral	ND	ND	Yes, complete eradication Yes, complete eradication Yes, but to a lesser extent	Electrostatic attraction possibly followed by fusion, protection of the antibiotic	*P. aeruginosa*	[52]
DPPC:PI	glucose oxidase alone or combined with horse radish peroxidase, chloroperoxidase or lactoperoxidase	0.1-0.22	Anionic	Yes	ND	Yes	Formation and release of antibacterial hydrogen peroxide and oxyacids directly into the biofilm	*Streptococcus gordonii*	[71, 72]
DPPC:Chol	Amikacin	0.3	Neutral	ND	Yes	Yes	Sustained release of the antibiotic near the infection site by triggered release (*in vivo* study, Arikace®)	*P. aeruginosa*	[51]
DPPC:DMPG	Tobramycin	0.23-0.4	Anionic	ND	ND	Yes	Sustained release of the antibiotic (*in vivo* study)	*B. cepacia*	[60]
DOTAP:DOPE:PC DOTAP:Chol:PC	Ciprofloxacin, Gentamycin, meropenem	0.1-0.15	Cationic	Likely	ND	Not for all the tested strains	Possibly sustained release of the antibiotic and/or fusion	*P. aeruginosa* *K. pneumoniae* *E. coli*	[65]
DPPC:Chol:DC-Chol	Benzyl penicillin (Pen G)	0.14	Cationic	Yes	ND	Yes, under certain conditions	Sustained release of the antibiotic	*S. aureus*	[73]
DMPC:Chol	Tobramycin, gentamicin, amikacin	0.35-0.4	Neutral	ND	ND	Not in the presence of mucin and CF sputum	Binding of the liposomes to mucin, alginate or sputum components	*P. aeruginosa*	[68]
DPPC:Chol DOPC:DPPG	Tobramycin	0.42 0.23	Neutral Anionic	ND	ND	No	EPS layer on the biofilm cells prevents	*B. cepacia complex*	[64]

							fusion Electrostatic repulsion		
DPPC:SA:Chol liposomes impregnated calcium sulfate	Gentamycin	ND	Cationic	ND	ND	Yes, complete eradication	Burst release followed by a prolonged sustained release (*In vivo* study)	*S. aureus*	[61]
DPPC:Chol liposomes impregnated porous β-tricalciumphosphate scaffold	Gentamycin sulfate	0.11-5.2	Neutral	ND	ND	Yes	Burst release followed by a prolonged sustained release (*In vivo* study)	*S. aureus*	[53]
PC:SA:Chol liposomes impregnated nano-hydroxyapatite/ β-tricalciumphosphate scaffold	Ceftazidime	0.16	Cationic	ND	ND	Yes	Burst release followed by a prolonged sustained release (*In vivo* study)	*S. aureus*	[54]
PC:SA:Chol liposomes impregnated nano-hydroxyapatite/chitosan/konjac glucomannan scaffold	Vancomycin	0.185	Cationic	ND	ND	Yes	Sustained release (*In vivo* study)	*S. aureus*	[55]
DSPC:Chol:ALN-TEG-Chol	Oxacillin	0.16 0.15	Anionic Anionic	ND	ND	Yes No	Fast release over several hours Low binding of these liposomes to the hydroxyapatite scaffold	*S. aureus*	[56]
DPPC:Chol:DSPE-PEG liposomes impregnated hydrogel	Ciprofloxacin	0.1	Neutral	ND	ND	ND	Prevention of bacterial adhesion	*P. aeruginosa*	[59]
PC:PS	Nisin	1	Neutral	ND	ND	ND	Inhibition of insoluble glucan	*S. mutans*	[57]

							synthesis for the prevention of caries		
DPPC:DPPA DPPC:DPTAP DPPC:DPTAP pectin coated	/	0.12-0.44	Anionic Cationic Anionic	ND	ND	ND	Protection against caries by adsorption of liposomes on teeth	/	[74]
DPPC:PI DPPG:PI DPPC	Triclosan	0.1	Anionic Anionic Neutral	Yes	ND	No Yes No	Only for low drug:lipid ratios sufficient targeting of the biofilm	*Staphylococcus epidermidis*	[75]
DPPC:Chol:SA	/	0.12	Cationic	Yes	ND	Likely	Targeting could lead to sustained release	*S. epidermidis*	[76, 77]
DPPC:Chol:SA DPPC:PI DPPG:DPPC DPPC:Chol:DDAB DPPC:DC-chol	Triclosan/Chlorhexidine Triclosan Triclosan / /	0.1	Cationic	Yes	ND	Yes No No ND ND	Targeting and sustained release	*S. epidermidis* *S. mutans* *Streptococcus sanguis*	[78]
DPPC:Chol:SA DPPC:Chol:DDAB DPPC:DC-chol	/ Vancomycin /	0.12	Cationic	Yes	ND	ND Yes ND	Targeting and sustained release	*S. aureus*	[79]
DMPC:Chol:DDAB DMPC:PI	Triclosan	0.12	Cationic Anionic	Yes	ND	No ND	Anionic liposomes fared better than cationic due to increased permeability for triclosan.	*S. sanguis* *Streptococcus salivarius*	[80]
DPPC:PI:DPPE-Anti-*S. oralis*	Chlorhexidine Triclosan	0.1	Anionic	Yes	ND	Yes	Targeting and sustained release	*Streptococcus oralis*	[81, 82]
DPPC:PI DPPC:Chol:SA DPPC:PI:DPPE-Anti-*S. oralis*	/	0.1	Anionic Cationic Anionic	Yes	ND	Likely	Targeting and sustained release	*S. epidermidis* *S. oralis*	[83]
DPPC:PI DPPC:PI:DPPE-Con-A DPPC:PI:WGA	/	0.75-0.11	Anionic	Yes	ND	Likely	Targeting and sustained release	*S. epidermidis* *S. sanguis* *Corynebacterium hofmanni* *Proteus vulgaris*	[84]
PC:Chol:SA	Metronidazole	2.9	Cationi	Yes	ND	Yes	Targeting and	*S. mutans*	[85]

PC:Chol:SA:Con-A			c Cationic or anionic depending on Con-A content			Yes	sustained release, protection against degradation		
DPPC:PI:Con-A DPPC:PI:WGA DPPC:Chol:SA DPPC:PI	Triclosan	0.1	Anionic Anionic Cationic Anionic	Yes	ND	Yes Yes Yes Yes	Targeting and sustained release	*S. gordonii* *S. epidermidis* *S. sanguis* *S mutans*	[86]
DPPC:PI:DPPE-Con-A DPPC:PI:DPPE	Triclosan	0.035-0.31	Anionic Anionic	Yes	ND	Yes ND	Targeting and sustained release	*S. sanguis* *S mutans*	[87]
DPPC:DOTAP:DSPE-PEG-WGA DPPC:DOTAP:DSPE-PEG DPPC:DPPG	Temoporfin	0.1-0.11	Anionic Neutral Anionic	Yes No No	ND	Yes (compared to the other 2 liposomes)	Targeting and increased delivery of temoporfin	*S. aureus* *P. aeruginosa*	[88]

2.2 Polymer and lipid-polymer hybrid nanoparticles

In contrast to the various beneficial traits described above, the use of liposomes can also have several disadvantages. The lipids show chemical instability such as hydrolysis of the ester bond and oxidation and peroxidation of the acyl chains. The composition of the lipids and the storage temperature play an important role in this. Furthermore, physical instability can lead to drug leakage during storage, which is especially problematic for liposomes with a low phase transition temperature, such as the fusogenic liposomes discussed in the previous section. Due to these limitations, polymer nanoparticles might constitute a valuable alternative. The polymeric particles already described in literature are typically formed from poly(lactic-co-glycolic) acid (PLGA) or chitosan or a mixture of PLGA and lipids to form so called lipid-polymer hybrid nanoparticles (LPH). [89, 90] PLGA is a biocompatible and biodegradable copolymer of lactic and glycolic acid and is FDA approved in various drug delivery systems. [91] Chitosan is a cationic, non-toxic, linear polysaccharide biopolymer and is obtained via deacetylation of chitin, the structural component of the exoskeleton of crustaceans. [92] Similar to the liposomes, these nanoparticles are biocompatible and biodegradable but less prone to chemical and physical instability. [69]

2.2.1 Controlled release

Polymer nanoparticles can be tailored to control the rate and duration of drug delivery. This can be useful to maintain high levels of antimicrobial agents inside biofilms, improving the anti-biofilm effect of the formulation. Gentamicin loaded PLGA nanoparticles were developed for the

treatment of *P. aeruginosa* infections. The formulation and the free drug were tested against biofilms *in vitro* as well as in a peritoneal murine infection model. The PLGA particles provided a sustained release of gentamycin, translating into a significantly enhanced anti-biofilm effect compared to the free drug. In the *in vivo* model, the free and PLGA encapsulated gentamicin were equally potent in clearing the infection but 96 hours after administering the formulations, the anti-biofilm effect of the free gentamicin was significantly reduced while biofilm formation was still largely inhibited by the nanoformulation. Control experiments showed that empty PLGA nanoparticles exhibited no antimicrobial effect against planktonic *P. aeruginosa* cells. [93] Cheow et al. compared the activity of a polymer and lipid-polymer hybrid (LPH) nanoformulation encapsulating levofloxacin against *P. aeruginosa* biofilms. [94] The polymer nanoparticles consisted of PLGA while the LPH nanoformulation consisted of a PLGA core covered with a lipid coat of phosphatidylcholine (PC) (Figure 3). Poly-(vinyl alcohol) (PVA) was added as a drying adjuvant to preserve the structural integrity of the particles during spray drying and to ensure good aerosolization characteristics of the nanoparticles.

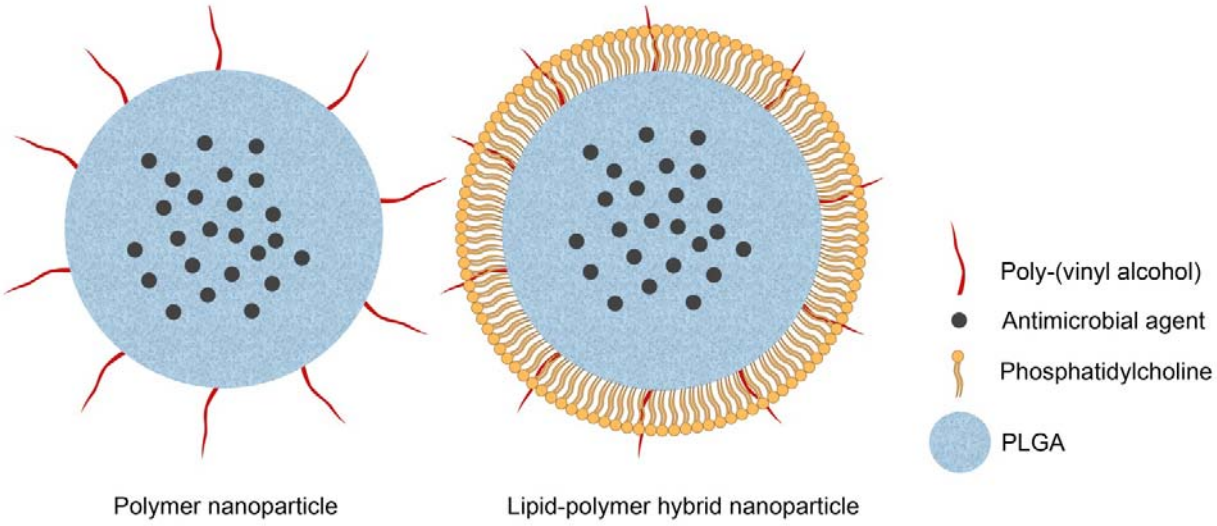

Figure 3: Schematic representation of polymer and lipid-polymer hybrid nanoparticles. Reprinted with permission from [94]

The LPH particles loaded with levofloxacin had a higher anti-biofilm efficacy compared to the polymer particles after 24 hours of antibiotic exposure. While the lipid coating of the LPH particles did not result in a higher affinity of these particles for the biofilm, it did slow down the release rate of the antibiotic from the particles. However, in a dose-kill experiment no significant impact of the level of antibiotic exposure on biofilm eradication was observed. This indicates that the release rate is not the cause for the observed superiority of the LPH nanoformulation. The difference is thought to originate from a better penetration into the biofilm matrix.

Formulations of ciprofloxacin and levofloxacin encapsulated in either PLGA or poly(caprolactone) (PCL) were evaluated in *E. coli* biofilms. [95] The antibiotics showed a higher encapsulation efficiency in PLGA, especially for the highly water soluble levofloxacin. Additionally, the PLGA particles showed a faster biphasic release profile compared to the PCL particles. This is important since the release profile can influence the anti-biofilm efficacy of a formulation. A fast burst release followed by a sustained release of the antibiotic is typically preferred. This is needed to prevent the increase in antibiotic tolerance of the surviving biofilm cells. [96] In a time-kill biofilm susceptibility test which was conducted over five days, *in vivo* conditions of drug removal were simulated by diluting the solution twofold every 24 hours. This test takes into account the effect of biofilm age and could reveal if the susceptibility of the biofilm is changed during a prolonged antibiotic exposure. The results showed that in the case of levofloxacin PLGA particles, resistant bacteria can survive the initial antibiotic treatment and will regrow the biofilm, even in the presence of levofloxacin above the MBIC. For the ciprofloxacin PLGA particles on the other hand, the growth of the surviving biofilm cells was inhibited over most of the five day period of the test, even for concentrations of ciprofloxacin as low as 1/16 of the MBIC. It was therefore concluded that PLGA encapsulated ciprofloxacin is more promising for the treatment of *E. coli* biofilms.

2.2.2 Antimicrobial polymers

Chitosan is another polymer that is used in drug delivery nanocarriers for anti-biofilm therapy. Chitosan itself has antimicrobial activity by adsorption onto the bacteria, causing aggregation and leakage of their intracellular content. Other mechanisms could be alteration of the cell permeability, binding with the bacterial DNA or chelation of trace metals which interferes with the production of virulence factors and bacterial growth. [97] The antimicrobial activity of chitosan was demonstrated against clinical isolates of *B. cepacia* complex. [92]

The antimicrobial activity of chitosan nanoparticles was evaluated against *S. mutans* biofilms. The nanoparticles were found to induce cell membrane damage, likely via direct interaction of the chitosan with the bacterial cells due to electrostatic interaction. These particles exhibit thus non-specific targeting to the biofilm cells. Cell membrane damage was more pronounced for chitosan with a lower molecular weight (below 200 kDa) than for higher molecular weight (above 500 kDa) chitosans (Figure 4). This is likely due to reduced diffusion of high molecular weight chitosan particles through the biofilm since for these particles no cell membrane damage was detected deeper into the biofilm. [98]

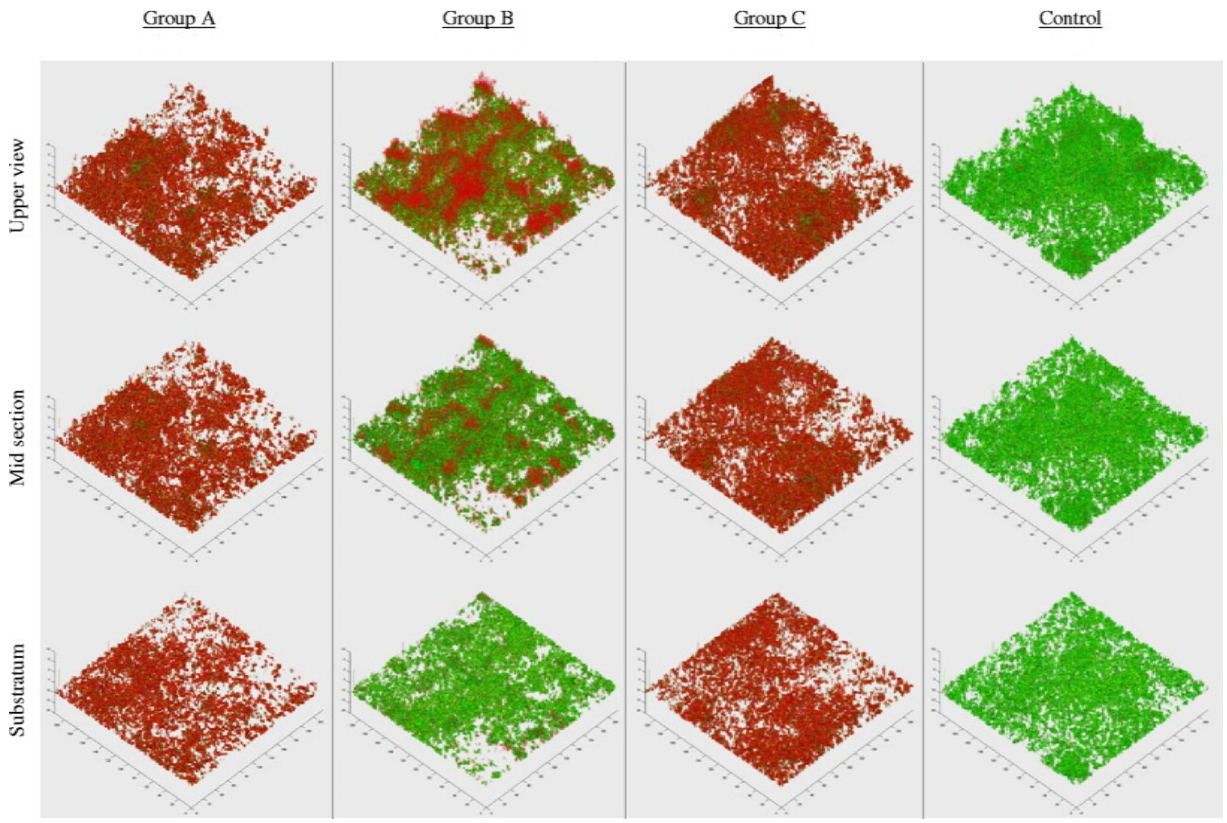

Figure 4: Antimicrobial action of chitosan nanoparticles on biofilms of S. mutans at different depths inside the biofilm. Green represents living biofilm cells and red dead cells. Group A and C are chitosans with a MW below 200 kDa and group B is a chitosan with a MW above 500 kDa Reprinted with permission from [98].

It is also possible to load antibiotics in chitosan derived nanoparticles. An example is the loading of vancomycin onto carboxymethyl chitosan (CMC) modified with folic acid (FA) and 2,2'-(ethylenedioxy)bis(ethylamine) (EDBE) by physical adsorption. [99] The presence of folic acid has proven to be essential for the antimicrobial activity of this vancomycin formulation. [100] The activity of this vancomycin nanoconjugate was evaluated against eight vancomcin susceptible or resistant *S. aureus* strains. The nanoconjugated vancomycin significantly decreased biofilm formation of all the tested strains, while the free vancomycin only decreased the biofilm formation of the vancomycin susceptible strains. The nanoconjugated vancomycin decreased the minimal inhibitory and bactericidal concentrations for planktonic cultures, delayed the onset of the stationary phase and reduced the production of virulence factors. The chitosan derived compound likely causes membrane depolarization which could lead to permeabilization of the outer membrane of the bacteria.

2.2.3 Encapsulation efficiency

Similar as for liposomes, the encapsulation efficiency of antibiotics in polymeric nanoparticles determines how much antimicrobial agent can be delivered to the biofilm. In the case of PLGA nanoparticles, several methods for improving the encapsulation efficiency have been described. The preparation of PLGA nanoparticles involves a nanoemulsion of a water phase and a water

immiscible solvent such as dichloromethane into which PLGA is dissolved. PLGA nanoparticles are formed by slow evaporation of the dichloromethane. A twofold increase in the encapsulation of highly water soluble antibiotics such as levofloxacin was obtained by either adding lecithin to the aqueous phase or by increasing the water miscibility of the oil phase. Lecithin, which has a phospholipid structure, adsorbs on the nanoparticle surface and is able to entrap levofloxacin. Increased water-miscibility of the oil phase speeds up the precipitation of the PLGA due to faster exposure to the aqueous phase, encapsulating the antibiotic before it can leak out. [101] A second way to increase the encapsulation efficiency in PLGA particles is the addition of helper hydrophilic moiety. One example is the addition of alginate that can increase the encapsulation efficiency of positively charged antibiotics by virtue of electrostatic interactions. These alginate containing particles showed a moderate cytotoxicity (20-30 % reduction of cell viability) on adenocarcinomic human alveolar basal epithelial cells at a concentration of 0.5 mg/ml. The cytotoxicity increased in a dose dependent manner.

[102] A third possibility is changing the pH at which the nanoparticles are formed. [93] For example, for the loading of gentamycin into the hydrophobic PLGA, increasing the pH from 5 to 7.4 allows deprotonation of the amino groups, thus reducing the hydrophilicity of gentamyin and enhancing its entrapment in the nanoparticles.

When using LPH nanoparticles, encapsulation of charged antibiotics can cause the nanoparticle formation to fail. To be able to encapsulate sufficient amounts of antibiotics, charge reversal of the lipid by adding counterionic surfactants has successfully been used. [103] Alternatively, one can complex the antibiotic with polyelectrolytes. [104] This complexation is fast, efficient, organic solvent free and does not require specialized equipment. Up to 80 % of the antibiotics were found to be incorporated using this complexation technique.

Table 2: Polymer and lipid-polymer hybrid nanoparticles tested for drug delivery to biofilms. Used abbreviations: PLGA: Poly(lactide-co-glycolic) acid, PC: Phosphatidylcholine, PCL: Poly(caprolactone), CMC: Carboxymethyl chitosan, EDBE: 2,2'-(Ethylenedioxy)bis(ethylamine), FA: Folic acid.

Nanoparticle composition	Antimicrobial agent	Size (µm)	Charge	Targeting to the biofilm?	Triggered release?	Improved antimicrobial effect?	Mechanism of action	Biofilm	Referer
PLGA	Carvacrol	0.21	Anionic	ND	ND	ND	Fluidification of the biofilm matrix	*S. epidermidis*	[105]
PLGA	Gentamycin	0.24-0.36	Neutral	ND	ND	Yes	Controlled/sustained release of antibiotic	*P. aeruginosa*	[93]
PLGA PLGA-PC	Levofoxacin	0.24-0.42	Anionic	No Yes	ND	Yes, but to a lower extent Yes	PLGA-PC particles could enhance antibiotic diffusion into the biofilm	*P. aeruginosa*	[94]

PLGA PCL	Ciprofloxacin Levofloxacin	0.17-0.24	ND	ND	ND	Yes with ciprofloxacin loaded PLGA as the most ideal	Biphasic release profile	*E. coli*	[95]
PLGA-PC	Calcein (as a model)	0.28	Anionic	ND	Yes	ND	Triggered release of the model compound by rhamnolipids	*P. aeruginosa*	[106]
Chitosan	/	0.02-1	Neutral	ND	/	Yes	Induction of cell membrane damage	*S. mutans*	[98]
CMC-EDBE-FA (Chitosan derivate)	Vancomycin	0.21-0.26	Cationic	Likely	ND	Yes	Induction of cell membrane damage	*S. aureus*	[99]
PLGA PCL/PLGA PCL	Levofloxacin	0.08-0.23	ND	ND	ND	Yes	Biphasic release profile	*E. coli*	[96]

3 Targeted delivery to biofilms

Functionalizing drug delivery nanoparticles with targeting ligands could be beneficial to achieve accumulation of the nanoparticles close to the bacterial cells and to promote close contact of the nanocarrier with the bacteria. Targeting can also be beneficial in environments with high shear forces, such as the oral cavity, where only short exposure times can be achieved (e.g. mouthwash). A distinction can be made between specific and non-specific targeting. Non-specific targeting mainly relies on charge based interactions and hydrogen bonding of the nanocarrier with the biofilm. Specific targeting is based on targeting ligands that selectively bind to a target molecule inside the biofilm. While biofilm targeting strategies have extensively been developed for liposomal drug delivery systems, to the best of our knowledge no studies regarding the specific targeting of polymer or lipid-polymer hybrid nanoparticles have been published to date.

3.1. Non-specific targeting

Jones and coworkers established that phosphatidylinositol (PI) and to a lesser extent DPPG caused the adsorption of liposomes to biofilms formed by bacteria recovered from the skin and the oral cavity. [75] It was found that the mole% of incorporated PI to obtain optimal interaction depends on the bacterial strain. [71, 75] They postulated that the hydroxyl content of the inositol and glycerol head groups of the lipids plays an important role, likely by the formation of hydrogen bounds with teichoic acids in the glycocalix of the bacteria. [107] Growth inhibition of *S. epidermidis* biofilms due to application of triclosan-containing DPPC:PI, DPPG:PI or DPPC liposomes was evaluated and linked to the drug to lipid molar ratio's. [75] All the liposomes tested showed a certain degree of inhibition of the bacterial growth, but only for low drug to

lipid ratios an advantage of liposomal delivery was seen. The targeting to the biofilm was less pronounced for high drug/lipid ratios, which could explain these results.

PI was also used for the passive targeting of so called 'reactive liposomes', i.e. liposomes containing enzymes that produce antibacterial substances in the presence of the right substrates. This strategy was evaluated for the treatment of *S. gordonii* biofilms. [71, 72] Glucose oxidase (GO) was incorporated into DPPC:PI liposomes, as well as GO combined with horse radish peroxidase (HRP), GO combined with chloroperoxidase (CPO) or GO combined with lactoperoxidase (LPO). The liposomes strongly adsorbed on the *S. gordonii* biofilms due to the presence of PI in the liposomes. Since the liposomes presumably remain intact, the antimicrobial action is caused by the formation of bactericidal substances such as hydrogen peroxide and oxyacids. These formed bactericidal substances diffuse out of the liposome and can act directly on the biofilm bacteria due to the close contact of the liposomes and the bacteria. This resulted in up to 50% inhibition of bacterial growth.

Stearylamine (SA) is another compound that can be used for the targeting of biofilm bacteria. In this case, the interaction is mainly based on the opposite surface charge of the SA containing liposomes and the bacterial membrane or negatively charged biofilm matrix compounds. Binding to matrix compounds might actually be disadvantageous since particles captured on matrix polymers cannot interact directly with the bacterial surface anymore. However, they can still serve as a depot for the release of antimicrobial agents in the direct vicinity of the biofilm. The amount of liposomes deposited in the biofilm was found to depend on the ionic strength of the surrounding medium, the temperature and the hydrophobicity of the bacteria. [76, 77] More liposomes are adsorbed at lower ionic strength, at higher temperatures and when the bacteria are more hydrophobic. It should be noted that although less liposomes adhere at lower temperatures, the liposomes adsorb more strongly, which is consistent with an electrostatic interaction. The SA content of the liposomes was observed to influence the adsorption profile of the liposomes. At a high concentration, SA is able to leak from the liposomes and binds to the bacteria, reducing the adsorption of the liposomes by reducing the number of binding sites available for the liposomes. [79]

In another report, the targeting capabilities of anionic PI bearing liposomes to *S. epidermidis* was compared to those of cationic SA bearing liposomes. [83] The SA bearing liposomes had a greater affinity to *S. epidermidis* biofilms than their PI bearing counterparts. Likely, the ionic interactions between the cationic liposomes and the anionic bacteria occur more efficiently compared to the hydrogen bonding interactions between the oligosaccharides in the glycocalyx of the bacteria and the anionic PI.

Cytotoxicity was reported for SA, likely limiting the use of this compound *in vivo*. [108, 109] As a less toxic alternative to SA, the cationic compounds dimethyldioctadecylammonium bromide (DDAB) or 3β-(N(N^1N^1-dimethylaminoethane) carbamoyl) cholesterol (DC-chol) can also target several skin and oral bacteria via electrostatic interactions. [76, 78-80] DC-chol containing liposomes showed the most effective targeting towards *S. epidermidis* while DDAB was the least effective targeting agent. The difference in targeting efficiency could be caused by the cationic nitrogen in the head group of DC-chol, which protrudes more from the liposome surface. [78]

DPPC:Chol:DC-chol liposomes delivering penicillin G to *S. aureus* biofilms were found to be more effective than the free drug when the overall drug concentration was lower and the exposure time shorter. A possible explanation is the complexation of penicillin G with the charged DC-chol of the liposome. At low penicillin G concentrations, the equilibrium between the free and complexed form of penicillin G is shifted towards the free form while at high penicillin G concentrations, the release of penicillin G is retarded. Over time, more and more liposomes adsorb onto the biofilm, increasing the penicillin G concentration in the biofilm and slowing penicillin G release from the liposomes. [73] Thus the interaction of the antimicrobial agent and the lipids can have an influence on the antimicrobial efficacy of the liposomal formulation.

Even though the DDAB bearing liposomes showed less effective targeting, approximately 60% of an *S. aureus* biofilm surface could still be covered by these liposomes. [79] Therefore, these liposomes could still increase the antimicrobial efficacy. DDAB bearing liposomes containing vancomycin were found to inhibit *S. aureus* growth more than free vancomycin for short (less than 30 minutes) incubation times. When using longer incubation times, the liposomes lose their advantage over free vancomycin, likely by disruption of the liposomes, which lowers the concentration of the antibiotic in close proximity to the biofilm cells. [79]

3.2 Specific targeting

Another targeting possibility is the use of immunoliposomes which carry covalently bound antibodies on the outer surface. These have the advantage of increased specificity and affinity compared to the aforementioned targeting strategies. The increased antibacterial action of immunoliposomes compared to the free antimicrobial agent is, like for other targeting compounds, the result of the retention of the immunoliposomes in the biofilm, which facilitates release of the antimicrobial agent in close proximity of the bacteria over longer periods of time.

The activity and affinity of immunoliposomes composed of DPPC, PI and the anti-*S. oralis* antibody conjugate of DPPE were tested against *S. oralis* biofilms *in vitro*. [81, 82] *S. oralis* is a commensal of the oral cavity and is able to cause opportunistic infections. In immunocompromised patients and patients with hematological malignancy, *S. oralis* can cause bacterial endocarditis, adult respiratory distress syndrome and shock. The immunoliposomes strongly adsorbed to *S. oralis* biofilms and showed decreased affinity to other oral commensal bacteria tested (*S. gordonii, S. sanguis* C104 and *S. salivarius* DBD and 8618), indicating that targeting of an antimicrobial agent to a specific organism can be achieved. The targeting was found to be independent of the charge of the lipids when comparing negatively charged DPPC:PI:DPPE-anti *S. oralis* immunoliposomes, positively charged DPPC:Chol:SA: DPPE-anti *S. oralis* and neutral DPPC:DPPE-anti *S. oralis* immunoliposomes. Likely, the antibodies mask the surface charge of the liposomes. [82] When comparing the antimicrobial effect of free and immunoliposome encapsulated chlorhexidine it was found that at low concentrations of chlorhexidine, the immunoliposomes were more effective in inhibiting growth of an *S. oralis* biofilm than the free drug. However, increased antimicrobial activity at low antimicrobial concentration was less apparent for immunoliposomes containing triclosan likely due to a difference in release profile of the lipophilic triclosan versus the hydrophilic chlorhexidine. [81] When comparing the affinity of DPPC:PI:DPPE-anti *S. oralis* immunoliposomes and liposomes of

the same composition but without the antibody, the non-targeted liposomes showed up to five times less affinity to the *S. oralis* biofilms. [82] Furthermore, the affinity of the DPPC:PI:DPPE-anti *S. oralis* immunoliposomes was compared to that of DPPC:Chol:SA cationic liposomes and DPPC:PI anionic liposomes. The cationic liposomes adsorbed best onto the biofilm, followed by the PI bearing liposomes. The immunoliposomes showed the lowest affinity. This can be explained by the fact that immunoliposomes only bind to specific antigens while the SA and PI bearing liposomes can adsorb electrostatically or via hydrogen bonding to many components in the biofilm. [82, 83]

As an alternative for antibodies, lectins can be used for the targeting of liposomes. One example is concanavalin A (Con-A), which selectively binds to α-mannopyranosyl and α-glucopyranosyl residues that can be found in the extracellular polysaccharide matrix of many biofilms. [110-112] This approach can again be used to target bacteria in the oral cavity, such as *S. mutans,* which is a significant contributor to dental caries. Increased binding of the targeted liposomes to *S. gordonii* or *S. mutans* biofilms was observed when comparing liposomes with and without Con-A. This Con-A mediated enhanced binding depends on the phospholipid composition of the liposomes and the surface density of Con-A. For example, the targeting to *S. mutans* by Con-A modified DPPC:PI:DPPE liposomes was less effective for increasing amounts of PI. [85, 87] When treating *S. gordonii* with triclosan, it was observed that Con-A modified liposomes carrying triclosan had an increased anti-biofilm efficacy compared to the free drug for short exposure times of the biofilm. [87] In a follow-up study, the anti-biofilm efficacy of Con-A modified PC:Chol:SA liposomes containing metronidazole was tested against *S. mutans* biofilms. Metronidazole encapsulated in untargeted liposomes led to a more pronounced growth inhibition than the free drug, likely due to protection of the drug from β-lactamases produced by the bacteria and/or by fusion of the liposomes with the bacteria. The use of lectin functionalized liposomes led to an increased biofilm growth inhibition compared to the use of the same liposomes without Con-A. [85] However, not all biofilm forming organisms are as effectively targeted with Con-A. Examples of organisms for which this targeting was less effective are *S. epidermidis* and *P. vulgaris*. [84, 86] This is likely due to the absence of the binding site for Con-A.

Another lectin often used as a targeting ligand is wheat germ agglutinin (WGA) that binds to N-acetylglucosamine and N-acetylneuraminic acid residues present in the extracellular matrix of many biofilms. [112, 113] Like for Con-A, not all organisms are as effectively targeted by WGA, with *P. vulgaris* again showing little liposome binding. [84, 86] Targeting of liposomes with WGA was successfully combined with photodynamic therapy. [88] The latter involves the use of a photosensitizer that produces reactive oxygen species (ROS) upon exposure to light. ROS are cytotoxic since these are able to oxidize proteins, nucleic acids and lipids. It was found that WGA modified DPPC:DOTAP:DSPE-PEG2000 liposomes were able to deliver more sensitizer to the bacterial cells compared to non-targeted liposomes. This resulted in the complete eradication of methicillin resistant *S. aureus* and an increased antimicrobial effect on *P. aeruginosa* bacteria in suspension.

4 Triggered release inside biofilms

Triggered release of the antibiotic from nanoformulations in close proximity to the biofilm bacteria is another approach to increase the local concentration of antibiotics in the biofilm. Generally speaking, the trigger can come from an external physical stimulus (e.g. electro-magnetic radiation, heat, ultrasound), or from the local biochemical environment (e.g. enzymes, virulence factors or pH changes). [114]

The only reported trigger in the context of biofilm treatment is the presence of rhamnolipids in *P. aeruginosa* biofilms (and CF sputum) that, due to their surfactant-like properties, could trigger the release of amikacin from DPPC:Chol liposomes. [51] Since the rhamnolipid concentration will be higher in close proximity of the biofilm, liposomes will release their content preferentially at the site of infection. A formulation of DPPC:Chol liposomes encapsulating amikacin for the treatment of pulmonary *P. aeruginosa* biofilms is currently being evaluated in phase 3 clinical trials.

The same concept was also evaluated for lipid-polymer hybrid nanoparticles composed of a PLGA core and PC coating. It was shown that rhamnolipids could trigger release from these formulations, but only for certain classes of encapsulated molecules. Levofloxacin and ofloxacin already showed a rapid release from the LPH particles in the absence of rhamnolipids. This was attributed to the fact that both drugs are able to pass lipid membranes. LPH particles loaded with calcein, a water soluble dye with a low permeability through lipid membranes, showed no significant release of calcein in the absence of rhamnolipids. Upon addition of rhamnolipids, a fast release of all the calcein could be observed. [106]

5. Studying the interaction and transport of nanoparticles in biofilms

Apart from testing a nanoformulation's anti-biofilm efficacy, it is important to obtain detailed information on how these nanoparticles interact with and behave in biofilms in order to rationally develop improved nanomaterials. This section presents an overview on the measurement and visualization of nanoparticle transport inside biofilms and the interaction of nanoparticles with biofilm components.

The ability of nanoparticles to penetrate into the biofilm is a first aspect that needs to be investigated. The nanoparticles likely penetrate into the biofilm by means of diffusion. In studies using fluorescent dyes, diffusion was found to be the main mode of transport inside biofilm clusters. [115-119] Two important parameters in this respect are the particle size and the surface characteristics of the nanoparticles. Good penetration into biofilms is essential to deliver the antimicrobial agent to all bacterial cells inside the biofilm. As highlighted above, the nanoparticles will often interact with the biofilm, either with the bacteria or with the biofilm matrix. On the one hand this is a disadvantage as it may hamper the distribution of particles throughout the entire biofilm. On the other hand it can be advantageous since the particles will

accumulate in the biofilm and form a depot for sustained drug release. In addition, if lipid-based nanoparticles bind to bacteria, fusion with the bacterial wall may be induced, allowing antibiotic delivery directly into the cytoplasm (see section 2.1.1). [20, 120] A classic way to determine the amount of liposomes adsorbed to the biofilm is to calculate the percentage apparent monolayer coverage (%amc). This is done by radioactive labeling of the liposomes and scintillation counting of a biofilm which is exposed to the liposomes and rinsed to remove non adhered liposomes. The %amc is calculated as the ratio of moles of lipid adsorbed to the biofilm to the theoretical number of moles of lipid that would be adsorbed if the biofilm was covered with a close-packed monolayer of liposomes. [75] While the %amc allows quantitative comparison between experiments, it should be noted that this method is unable to distinguish between liposomes adsorbed to the bacteria and the biofilm matrix. Microscopy experiments can provide complementary information in that sense.

To observe in detail the interaction of liposomes with bacterial cells, transmission electron microscopy (TEM) is often used since it allows the observation of subcellular features such as the bacterial membrane. Malcolm Jones and coworkers have extensively investigated the adsorption of liposomes on bacterial biofilms using TEM to visualize the binding of targeted liposomes to *S. epidermidis* or *S. oralis* biofilms. [83] They studied the interaction of phosphotungstenic acid labeled DPPC:PI liposomes or DPPC:Chol:SA liposomes with *S. epidermidis* biofilms. Phosphatidylinositol and stearylamine are known targeting ligands for several oral and skin associated bacteria (see also 3.1). [75, 76, 84, 86] It was observed with TEM that both types of liposomes cluster around the bacteria in a similar way. [83] They also studied the binding of anti-*S. oralis* immunoliposomes and found that the immunoliposomes were located on the surface of the bacterial cells with a preference for the septal region between dividing cells. [83]

Later, Jones and coworkers established a protocol to investigate the adsorption of fluorescent liposomes to immobilized biofilms using confocal microscopy. [121] Confocal microscopy has the advantage over TEM that it offers the possibility of observing living, fully hydrated biofilms, while requiring less extensive sample preparation. However, confocal microscopy has a substantially lower resolution than TEM and does not allow to resolve the liposome-biofilm interface in great detail. TEM and confocal microscopy can thus be considered as complementary techniques. Jones et al. added anionic, cationic and PEG modified cationic liposomes to an *S. aureus* biofilm grown in culture chambers suited for optical microscopy. Confocal imaging revealed that cationic liposomes were not able to penetrate into the biofilm, but rather accumulated on the surface of the biofilm in a 20 µm thick layer. The anionic liposomes on the other hand did neither penetrate in nor adsorb to the biofilm, which was attributed to repulsion by the negative surface charge of the bacteria. [122] The pegylated cationic liposomes showed reduced adsorption onto the biofilm. This is expected since pegylation reduces non-specific interactions of the liposomes with biological materials. [123] In a follow up study, the influence of the liposomal zeta potential on the strength of their adsorption on an *S. aureus* biofilm was investigated using confocal microscopy. [122] Fluorescently labeled cationic liposomes of similar size but with a zeta potential ranging from 1 to 41 mV were added to an *S. aureus* biofilm in a flow cell. The critical shear stress required to

completely remove the adsorbed liposomes from the biofilm increased slightly but not significantly with increasing zeta potential.

Meers et al. studied the penetration of liposomes in bacterial clusters of *P. aeruginosa* biofilms using confocal microscopy. [51] Neutral, fluorescent DPPC:Chol liposomes of about 0.3 µm in diameter were synthetized and added to a the biofilm in a flow cell together with 1 µm fluorescent polystyrene beads. The liposomes seemed to accumulate in the periphery of the cluster while in the center, the concentration appeared to be the same as in the fluid surrounding the biofilm. The 1 µm polystyrene particles on the other hand did not significantly penetrate into the bacterial cluster. It was observed before that the size of solutes and nanoparticles influences their diffusion coefficient and penetration into biofilms. [124]

Confocal microscopy was also the method of choice of Miller et al. to investigate the deposition of pegylated L-tyrosine polyphosphate (LTP) microparticles onto *P. aeruginosa* biofilms in a flow cell. [125] Z-stacks were recorded after infusion of the fluorescently labeled LTP nanoparticles with an average diameter of 1.2 µm. The particles were mostly located at the fluid-biofilm interface up to 2 hours after deposition. After 4 hours, the particles were distributed more evenly throughout the biofilm. Due to their adherence to the biofilm, the final concentration of LTP nanoparticles was estimated to be 2 orders of magnitude higher inside the biofilm as compared to the starting suspension.

More advanced fluorescence microscopy methods can be used to measure diffusion coefficients in different locations of a biofilm. One of these techniques is fluorescence correlation spectroscopy (FCS). By measuring fluorescence fluctuations caused by fluorescent particles or molecules diffusing in and out the focused laser beam of a confocal microscope, their local diffusion coefficient can be calculated. [126] This technique was applied to measure the diffusion of fluorescent polymer nanoparticles (57, 92 and 135 nm in diameter) in *P. fluorescens* biofilms. [127] By varying the growth conditions of the bacteria, either biofilms consisting of dense clusters or loose biofilm flocks were grown. The particles showed little penetration in dense biofilms while in loose biofilms, a steep decrease in the diffusion coefficient was observed for larger nanoparticles. Comparison with the diffusion of differently sized fluorescent molecules revealed an estimated pore size of 50 nm for a loose biofilm while this decreased below 10 nm for a dense biofilm. In another study, FCS revealed that the hydrophobicity of *Lactococcus lactis* cells affected the diffusion of 50 nm anionic carboxylate modified polystyrene nanoparticles through the biofilm matrix. [128] The more hydrophobic the surface of the cells, the lower the fraction of freely diffusing nanoparticles.

Single particle tracking (SPT) microscopy is another method to measure the diffusion coefficient of nanoparticles inside biofilms. In SPT, a time-lapse video of the movement of the nanoparticles inside the biofilm is recorded from which the motion trajectories of individual particles are calculated using image processing. From the trajectories, the diffusion coefficient and mode of motion (free diffusion, anomalous diffusion or directed motion) can be derived on a particle by particle basis. [129] SPT was applied to study the diffusion of polystyrene nanoparticles with different surface modifications in living, hydrated *Burkholderia multivorans* and *P. aeruginosa* biofilms. [130] These two pathogens are able to cause chronic pulmonary

infections in CF patients. Although *B. multivorans* infections occur less frequent, the increased virulence compared to *P. aeruginosa* causes the lung function to decline more rapidly. Both anionic and cationic beads were largely immobilized to biofilm components, reducing their average diffusion coefficient considerably. Pegylated beads on the other hand were nearly as mobile as in water (Figure 5). Confocal images showed that positively charged nanoparticles attached to wire-like compounds of the biofilm matrix, likely biofilm matrix polymers and extracellular DNA (eDNA). Unexpectedly, the negatively charged particles were observed to bind in close proximity to the bacteria (Figure 6). Also other interactions such as hydrophobic interactions between the polystyrene and the bacteria were likely involved in overcoming the electrostatic repulsion with the negatively charged cell membrane.

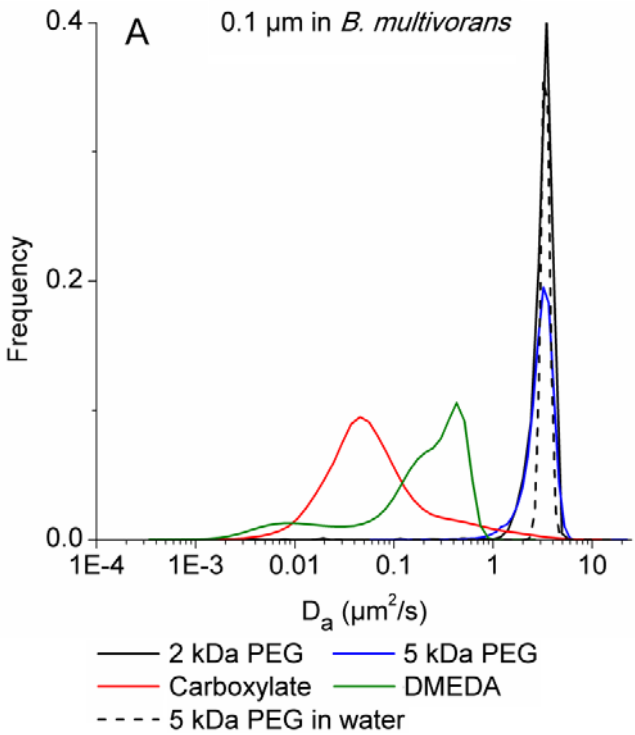

Figure 5: Diffusion coefficients of the modified 0.1 µm polystyrene nanoparticles in B. multivorans biofilms. The diffusion of the charged nanoparticles is considerably lower compared to pegylated particles. [130]

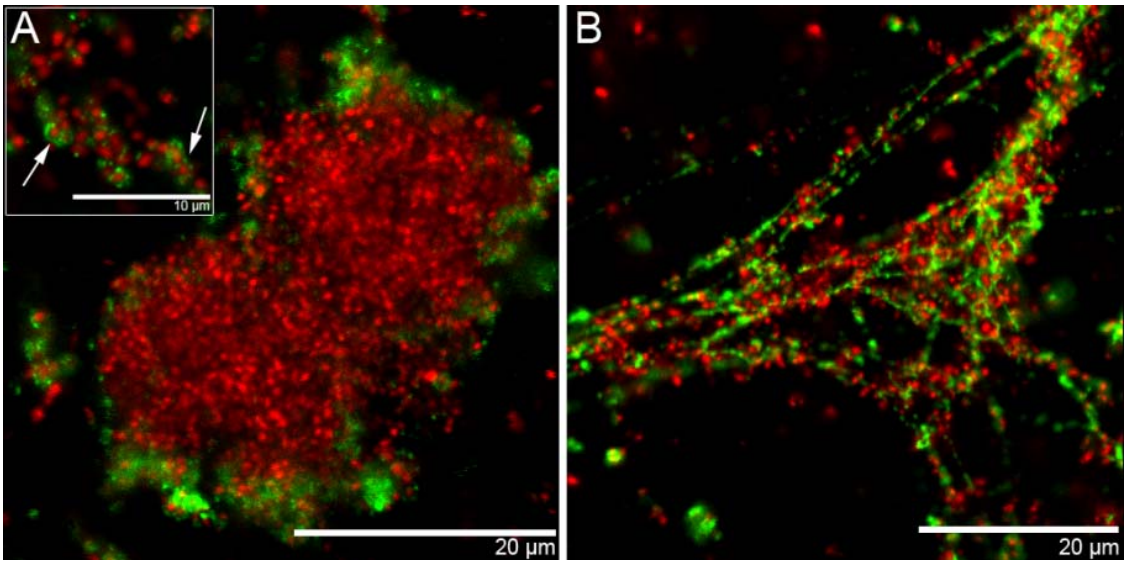

Figure 6: (A) Anionic polystyrene nanoparticles (green) bind in close proximity of B. multivorans bacteria (red). The arrows in the insert show bacteria surrounded by particles. (B) Cationic polystyrene nanoparticles (green) attached as beads on a thread to matrix polymers surrounding the bacteria (red). [130]

In a follow up study, the transport of 0.2 µm positively and negatively charged fluorescent polystyrene nanoparticles was evaluated in 5 different *B. cepacia* complex strains in the presence or absence of DNase. In the absence of DNase, both anionic and cationic particles strongly interacted with all the tested biofilms, resulting in diffusion coefficients up to 10 times lower compared to water. However, when the biofilms were grown in the presence of DNase to break down eDNA, the diffusion coefficient of the positively charged nanoparticles increased approximately twofold. [64]

6. General discussion and future outlook

Biofilm formation plays a major role in the persistence of bacterial infections as cells in biofilms are able to evade host immune defenses and show increased antibiotic resistance. Little success has been achieved in treating biofilm infections with classical antibiotic therapy and innovative ways to deliver antimicrobial substances in a sufficiently high concentration to the biofilm bacteria are expected provide therapeutic benefit. The ability of liposomes and polymer nanoparticles to deliver antimicrobial substances into biofilms has been extensively studied in the past decade. As reviewed here, many researchers have confirmed improved anti-biofilm activity of lipid and polymer-encapsulated antibiotics.

Mechanisms behind the increased activity of nanoparticle incorporated antibiotics include fusion of liposomes with the bacterial cells, protection of the antibiotic against degrading enzymes, prevention of inactivation by binding to components surrounding the biofilm, increased transport of the nanoformulation into the biofilm and increased contact time between the bacteria and the antibiotic. For non-targeted nanoparticles, liposomes with

fusogenic properties are preferred since they are able to deliver the antimicrobial agent through the outer membrane of the bacteria. In this way, high doses of antimicrobial agents can be delivered directly into the bacteria. An additional advantage of fusogenic liposomes is their flexible membrane which in some cases can aid in the transport of the liposomes to the infection site. Apart from liposomes, also polymer and lipid-polymer hybrid nanoparticles have been evaluated. Although positive results have been reported, a comparative study of the antimicrobial activity of liposomes, polymer and lipid-polymer hybrid nanoparticles is currently lacking. Several factors contribute to the overall antimicrobial efficacy of a nanoformulation, such as the release profile, the ability of the nanocarrier to interact with the biofilm, the occurrence of fusion or release of the antimicrobial agent under influence of substances secreted by the bacteria. All of these factors would need to be investigated to clarify why a certain formulation fares better than the other. Having this information would contribute to the development of even more efficient nanoformulations.

Targeting of the nanoparticles to the biofilm is an interesting approach to achieve longer contact times and the delivery of the antibiotic in close proximity of the bacterial cells. Adding PI and SA as non-specific targeting moieties to liposomes resulted in an increased adhesion to biofilms as compared to the use of immunoliposomes. If this actually improves anti-biofilm therapy remains to be demonstrated. However, one can envisage that the use of immunoliposomes is advantageous when a certain type of bacteria needs to be targeted (e.g. dental caries or periodontal pocket disease) or when the biofilm resides in a complex biological medium (e.g. lungs of CF patients). Clearly further studies are needed, both *in vitro* and *in vivo*, to fully assess the potential of immunoliposomes. Lectin binding Con-A and WGA are interesting alternative ligands to achieve nanoparticle targeting to biofilms. As they are less expensive than antibodies, they could be attractive when targeting to a specific bacterial species is not required, although not all biofilms contain the target for Con-A and WGA. Comparative studies of the anti-biofilm efficacy and the specificity of liposomes targeted using these lectins or antibodies would be useful.

The addition of substances that modify the biofilm structure or the surrounding medium could improve the anti-biofilm efficacy of nanoparticle delivered antimicrobial agents. For example, alginate lyase can degrade the matrix of a mucoid *P. aeruginosa* biofilm or DNase can degrade extracellular DNA in the biofilm matrix or in cystic fibrosis mucus. [68] Another biofilm structure modifier is carvacrol, a component of essential oils which has a wide spectrum of antimicrobial activity. Carvacrol encapsulated in PLGA nanocapsules significantly influenced the elastic and viscous moduli of *S. epidermidis* biofilms compared to free carvacrol as measured by a cone-plate rheometer, indicating fluidification of the biofilm matrix. [105] Possibly, this approach could increase the penetration of antimicrobial agents into the deeper regions of the biofilm where persisters are believed to reside.

It should be noted that not in all studies an improved efficiency of nanoparticle encapsulated antibiotics was observed. It is important to find a balance between protecting the encapsulated drug and being able to release the drug near the bacteria. Triggered release of the antibiotic from the particles could be a complementary strategy to overcome biofilm resistance in this respect. By suddenly releasing high amounts of antibiotics in close proximity to the bacteria, the

upregulation of resistance mechanisms may be prevented. Rhamnolipids, a surfactant-like virulence factor of *P. aeruginosa*, have proven to be an efficient endogenous trigger for release of antimicrobial agents from liposomes and lipid-polymer hybrid nanoparticles. Arikace®, a liposomal formulation of amikacin which shows triggered release upon contact with rhamnolipids, is in final stages of clinical trials for treating *P. aeruginosa* infections in cystic fibrosis. Other physical or chemical triggers could be explored in the future, such as pH changes, enzymes present at the infection site, heat, electromagnetic radiation and ultrasound.

Although a lot of research is focused on the antimicrobial efficacy of drug delivery nanocarriers, only few studies are looking into the transport and penetration of nanoparticles inside biofilms. Advances in optical imaging techniques allows to study the interactions of these nanoparticles with the biofilm as well as their capability to penetrate into the biofilm. Confocal microscopy, SPT and FCS all have proven to provide valuable information. Electron microscopy serves a complementary role as it can provide direct evidence for fusion of liposomes with bacteria. By comparing different nanocarriers, the properties that can lead to optimized drug delivery can be identified as well.

The present overview indicates that the use of nanoparticles for anti-biofilm therapy is a viable approach. However, the majority of papers discussed in this review are based on *in vitro* observations. Since the *in vitro* environment is far less complex than the *in vivo* situation, future work needs to be directed towards *in vivo* studies. In this context, it is important to mention that the *in vitro* cytotoxicity of the nanoformulations is only rarely tested on eukaryotic cells. Although many lipid and polymer nanoparticles are regarded as being biocompatible, cytotoxicity towards eukaryotic cells can still occur and is a factor that needs to be taken into account, preferably before *in vivo* studies are undertaken. Also, information about the relative partitioning of the nanoparticles between the biofilm bacteria and eukaryotic cells could be useful in this respect. The information gathered from *in vitro* experiments now allows the rational design of nanocarriers for *in vivo* treatment of biofilm infections. Special attention will need to be paid to adapting the formulations to the chosen delivery route while still retaining the characteristics necessarily for increased delivery of antibiotics to the biofilm. The complexity of the situation likely explains why so little companies are running clinical trials with nanomedicines for the treatment of biofilm infections. In an era which is increasingly dominated by resistant bacterial strains, it is paramount to make the transition from *in vitro* to *in vivo* studies as fast as possible.

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