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Nano-fats for bugs: the benefits of lipid nanoparticles for antimicrobial therapy

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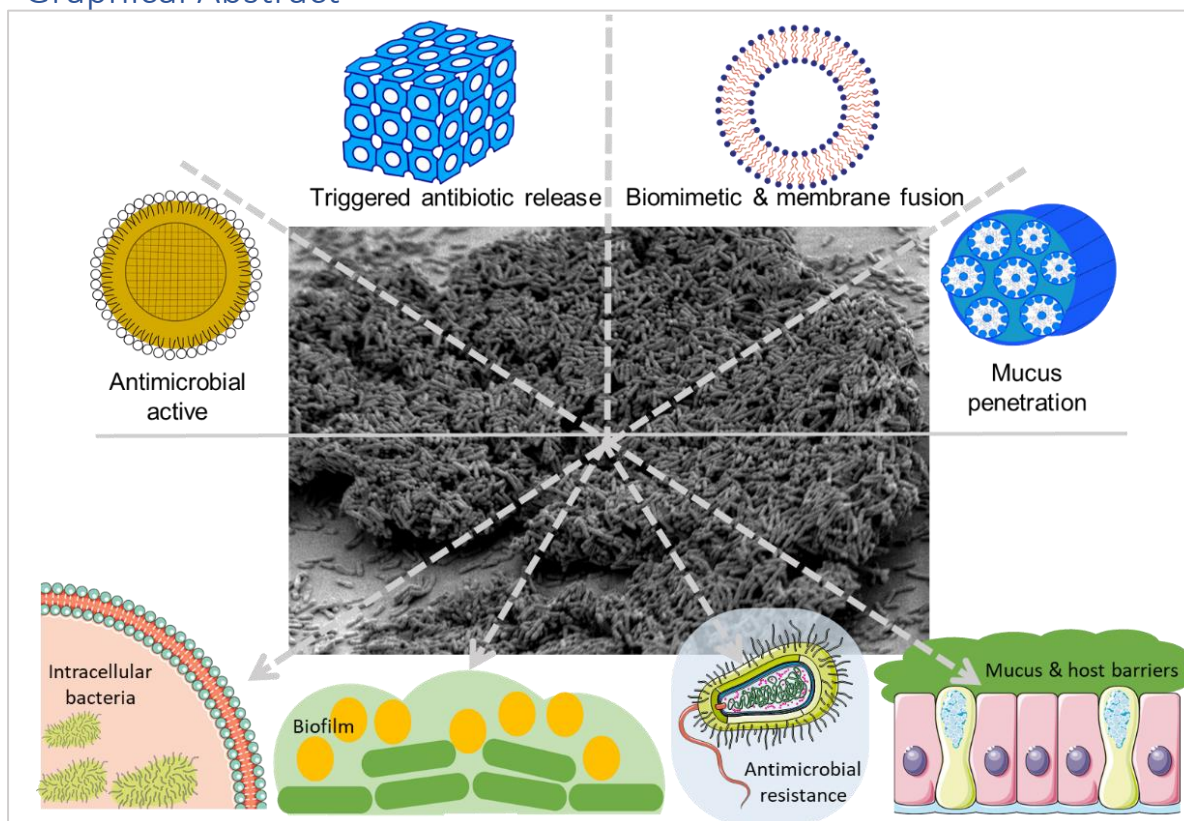
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Keywords: Antibiotics, antimicrobial resistance, biofilms, intracellular infections, lipid-based drug delivery, liposomes, lipid nanoparticles, liquid crystalline nanoparticles

Abstract

Bacterial infections are an imminent global healthcare threat evolving from rapidly advancing bacterial defence mechanisms that antibiotics fail to overcome. Antibiotics have been designed for systemic administration to target planktonic bacteria, leading to difficulties in reaching the site of localised bacterial infection and an inability to overcome the biological, chemical and physical barriers of bacteria, including biofilms, intracellular infections and antimicrobial resistance. The amphiphilic, biomimetic and antimicrobial properties of lipids provide a promising toolbox to innovate and advance antimicrobial therapies, overcoming the barriers presented by bacteria in order to directly and effectively treat recalcitrant infections. Nanoparticulate lipid-based drug delivery systems can enhance antibiotic permeation through the chemical and physical barriers of bacterial infections, as well as fuse with bacterial cell membranes, release antibiotics in response to bacteria and act synergistically with loaded antibiotics to enhance the total antimicrobial efficacy. This review explores the barriers presented by bacterial infections that pose bio-pharmaceutical challenges to antibiotics and how different structural and functional mechanisms of lipids can enhance antimicrobial therapies. Different nanoparticulate lipid-based systems are presented as valuable drug delivery systems to advance the efficacy of antibiotics, including; liposomes, liquid crystalline nanoparticles, solid lipid nanoparticles, nanostructured lipid carriers and lipid nanocarriers. In summary, liquid crystalline nanoparticles are emerging with the greatest potential for clinical applications and commercial success as an “all-rounder” advanced lipid-based antimicrobial therapy that overcomes the multiple biological, chemical and physical barriers of bacteria.

Graphical Abstract



1. Introduction

Bacteria contribute to one of the greatest causes of death worldwide – infectious diseases [1]. As adaptive organisms, the virulence and fitness of bacteria are rapidly evolving, intensifying the critical healthcare issue of infectious diseases [2, 3]. Bacteria have three crucial mechanisms that control their virulence and survival, i.e. biofilm formation, intracellular survival and antimicrobial resistance. These mechanisms are associated with numerous biological, chemical and physical barriers preventing effective antimicrobial therapy. As a result, persistent, lifelong infections are highly prevalent in the clinic, e.g. in cystic fibrosis (CF), pneumonia, rhinosinusitis, non-healing chronic wounds, and urinary tract infections [4, 5].

There is an urgent need to develop innovative antimicrobial therapies against these persistent bacterial infections. While efforts have primarily focused on developing new chemical entities, re-purposing and re-formulating compounds already available is considered a faster strategic approach in the current urgent situation. Consequently, the development of new antimicrobial therapies has recently shifted towards improving the delivery of commercially available antibiotics by using advances in nanotechnology to direct antibiotics to the site of infection. Drug delivery systems based on polymers, inorganic materials, metals, lipids, and composites of synthetic and natural materials, are all reported to improve the performance of existing antimicrobial compounds [6-8].

Drug delivery systems can address the various challenges faced by antibiotics, including bypassing antimicrobial resistance mechanisms such as enzymatic inactivation and restricted permeation and

overcoming multiple chemical and physical barriers that limit the access of antibiotics, such as biofilm and intracellular infections [9-11]. While drug delivery technologies cannot directly overcome the modifications induced by resistant bacteria associated with the molecular target of the antibiotics, they can overcome the physicochemical barriers from the bacteria and host, exposing bacteria to therapeutically appropriate antibiotic concentrations through directed and targeted therapy.

Lipids are particularly promising building blocks for the design of drug delivery systems. With inherent antimicrobial properties and the ability to self-assemble into structural vehicles for drug delivery [12, 13], amphiphilic lipids serve as a promising tool and solution to innovate and advance antimicrobial therapy against recalcitrant bacterial infections. Amphiphilic lipids are unique biologically active molecules containing both hydrophobic and hydrophilic portions with properties that drive specific structural and functional characteristics mimicking the chemistry of cellular vesicles. As biocompatible and biomimetic drug delivery systems, lipid-based drug delivery systems have been highly prominent as potential delivery systems for antibiotics [6]. This review will focus on the biological, chemical and physical barriers associated with bacterial infections that pose significant challenges to antimicrobial therapies and details how lipid-based drug delivery systems can overcome these. Through the following sections, we provide an updated perspective on the use of lipids as antimicrobial compounds and delivery systems, specifically liposomes, liquid crystalline nanoparticles, solid lipid nanoparticles, lipid nano-carriers, and nanostructured lipid carriers. The discussion is divided across four principal advantages that lipid-based nanoparticles offer antibiotics, namely (1) innate and synergistic antimicrobial effects, (2) fusion with bacterial membranes, (3) attractive particulate surface characteristics, and (4) bacterial triggered antibiotic release.

2. Bacterial barriers: biofilms, intracellular infections and antimicrobial resistance

Survival is the primary focus of bacteria. Through creating barriers that protect, nourish and sustain the microbe's lives, bacteria can adaptively withstand most environments within the human body. Biofilms, intracellular survival and antimicrobial resistance are key examples of advanced survival strategies (Figure 1) [3, 14]. Biofilm fortresses or the intracellular space of crucial immune effector cells protect bacteria from noxious stimuli, while advanced communication between bacteria nourishes their survival and encourages the development of new defensive strategies [14, 15]. The barriers created by bacteria lead to highly pathogenic, persistent and detrimental diseases in humans. Through understanding the underlying defensive strategies of bacteria, better antimicrobial therapies can be developed.

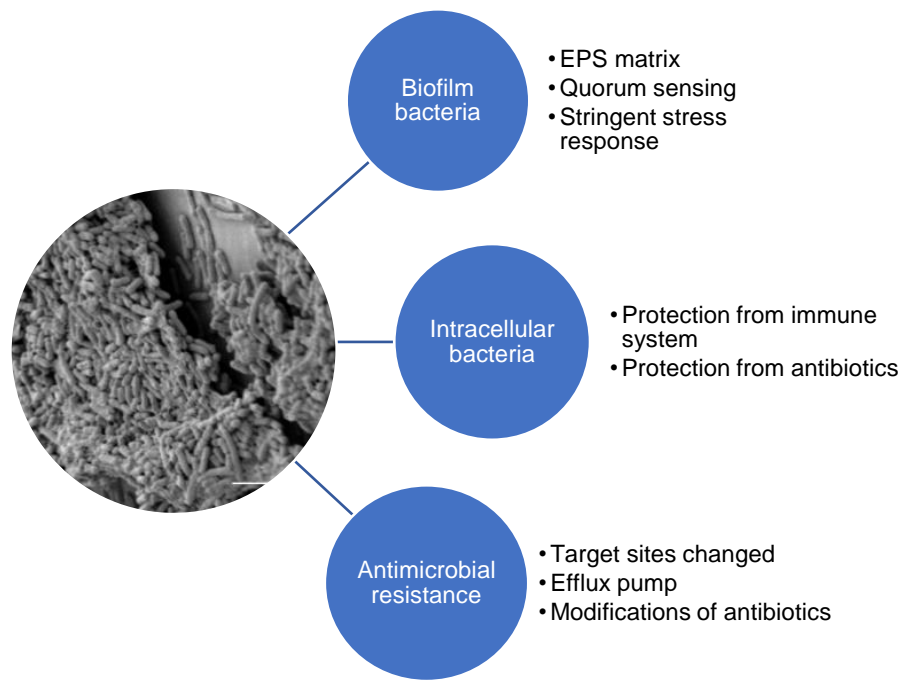


Figure 1. Bacterial mechanisms that create biological, chemical and physical barriers, and limit effective antimicrobial therapy

2.1. Biofilms

Biofilms are syntrophic clusters of microorganisms, adhered to biotic or abiotic surfaces and protected by a self-produced matrix. The notion of bacteria living as predominantly free-floating, planktonic cells was rebuffed by Costerton and colleagues [16], upon finding that bacteria predominately existed in these complex communities of biofilms (Figure 2). The bacteria in the biofilm are phenotypically different from those in the planktonic state, harbouring significantly higher virulence, and can tolerate up to 1000-fold higher concentrations of conventional antibiotics [4, 16]. The protective, extracellular polymeric substance (EPS) matrix is comprised of extracellular DNA, proteins, nucleic acids, and polysaccharides, which maintain the intimate environment of bacteria and restricts access to the bacteria [14]. The phenotypically different inner bacterial community have lower metabolic states that reduce their susceptibility to antibiotics, which typically kill or inhibit bacteria when in the metabolically-active, planktonic state [17]. In humans, biofilms cause persistent and chronic tissue-related infections, e.g. from *Pseudomonas aeruginosa* (*P. aeruginosa*) colonisation in the lungs, sinuses or wounds and implant-related infections, and from *Staphylococcus aureus* (*S. aureus*) contaminating joint prosthetics and catheters [18-20]. The syntrophic consortium of bacteria in biofilms is a fortress with advanced strategies to withstand the effects of antibiotics.

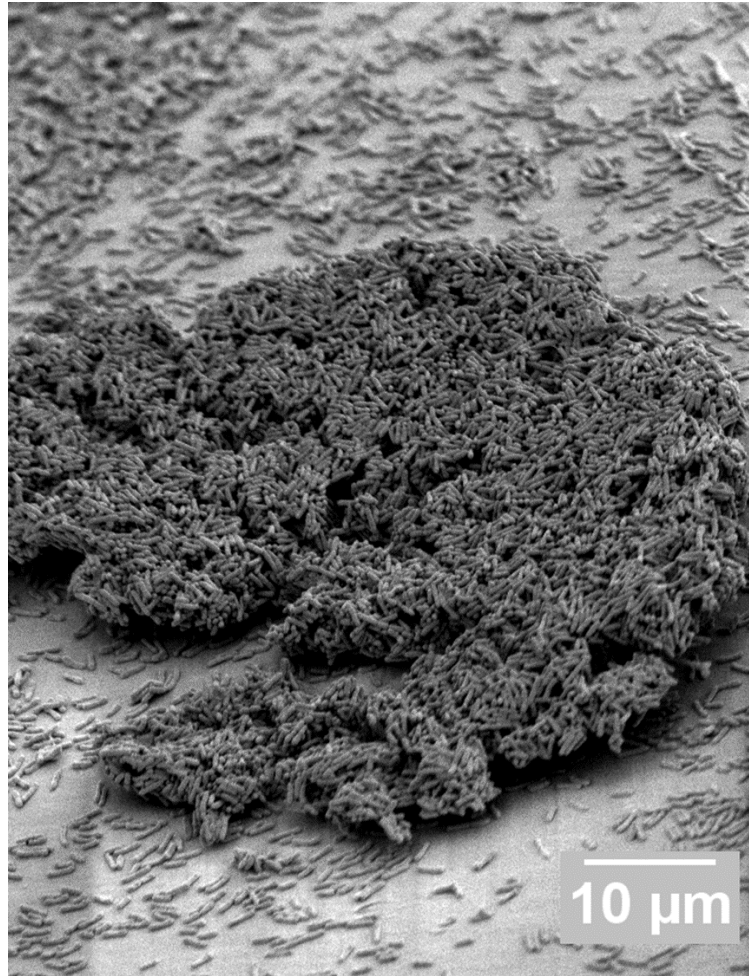


Figure 2. Scanning electron micrograph of *Pseudomonas aeruginosa* biofilm surrounded by planktonic bacteria attached to polystyrene pegs.

Biofilms are underpinned by a host of impressive stress responses and quorum sensing abilities, enabling tightly coordinated responses and exerting dominance in any environment. The “stringent stress response” is a vital stress-coping mechanism from the accumulation of secondary-messenger nucleotides, and it diverts cellular energy away from growth and division so the biofilm can withstand environmental stressors [21-23]. The stringent stress response also activates quorum sensing, leading to the tightly controlled communication through genetic regulation in the dense population of bacteria in the biofilm [24]. Quorum sensing further results in a multitude of virulence factors, for example, the production of specific chemicals to cause host damage such as LasB elastase in *P. aeruginosa* [25-27]. The social cooperation within biofilms enables strategic accumulation of resources, with the primary goal of survival [14]. Overall, the unwavering dominance of biofilms leads to their highly pathogenic and persistent role in human infectious diseases.

2.2. Intracellular infections

In tissue-related infections, bacteria typically survive in the extracellular space, growing on epithelial surfaces to form biofilms and stimulate an aggravated host immune response [28]. The host up-regulates an inflammatory response to defend itself against the bacteria, with mononuclear phagocytes

(i.e. monocytes and macrophages) acting as the key effector cells. To withstand the effects of the inflammatory response, bacteria can also conceal and protect themselves in these critical effector cells of the immune system. Bacteria survive in the intracellular space of mononuclear phagocytes by diverting the endosomal pathway, entering directly into the host cells' cytoplasm [29]. *S. aureus* and *Mycobacterium tuberculosis* (*M. tuberculosis*) are organisms renowned for their ability to cause intracellular infections. Inside professional and non-professional phagocytes, bacteria are concealed from the innate immune system, evading attack from antibodies, antibiotics and obtain a direct supply of nutrients that would have otherwise been restricted [5, 30]. Intracellular infections are persistent and difficult to detect and treat due to the bacteria utilising the host's barriers to conceal themselves, including the hydrophobic epithelial barrier that may have a heterogenous coating of mucus.

2.3. Antimicrobial resistance

Beyond these innate growth and survival mechanisms of bacteria, antimicrobial resistance is a highly adaptive response by bacteria to protect themselves from antibiotics. Antimicrobial resistance is a global healthcare crisis that has evolved from inappropriate and excessive use in humans and animals, enabling bacteria to develop mechanisms to overcome the effect of many antibiotics. The leading cause of nosocomial infections are resistant to multiple antimicrobial therapies, collectively known by the acronym ESKAPE Pathogens (e.g. *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter* spp., *P. aeruginosa* and *Enterobacter* spp.) [31]. These notorious pathogens create catastrophic and fatal disease due to their sophisticated resistance mechanisms [32] and are causing a catastrophic healthcare crisis.

The increased selective pressures of improper and excessive antibiotic use promote the biological responses that improves the survival of bacteria in the presence of otherwise harmful substances. Antimicrobial-resistance mechanisms include the ability of bacteria to; modify antibiotics, express efflux pumps to prevent the entry of antibiotics or to actively remove antibiotics from the intracellular space, and alter the target sites of antibiotics [33]. These mechanisms are highly specific concerning the bacterial species and antibiotics used, but inhibit the effectiveness of most of the first-line antibiotics available today [34]. Antimicrobial resistance is a leading contributor to deaths worldwide, and within the next 30 years, the predicted global annual death toll from antimicrobial drug-resistant infections is 10 million deaths per year [35]. The global catastrophe demands alternations in the way antibiotics are used.

3. Challenges in the development of antimicrobial compounds

While biofilm formation and intracellular survival are innately inscribed in bacteria, antibiotics were designed to kill single (planktonic) colonies of bacteria, without an understanding of biofilms and intracellular survival. Altogether, biofilms, intracellular survival and antimicrobial drug resistance create several biological, chemical, and physical barriers to effective antimicrobial therapy. The biological barriers include the advanced mechanisms in biofilm formation (i.e. quorum sensing and the stringent stress response) and antimicrobial resistance (i.e. efflux pumps and target modifications). The chemical barriers include the protective matrix of the biofilm, and antibiotic modifications from antimicrobial

resistance and the physical barriers include the host cell membrane and up-regulated host responses (i.e. mucus production and inflammation). Antibiotics cannot target these mechanisms or lack physicochemical properties to bypass these barriers of bacteria, leading to significant treatment challenges.

3.1. Limitations of the physicochemical properties

The physicochemical properties of drugs affect their trajectory after administration. While drugs need to be soluble in bodily fluids to cause an effect at the target tissue, they need reasonably high partition coefficients to pass through hydrophobic and biological barriers. While many antibiotics are water-soluble, their permeability across biological barriers including the bacterial cell envelopes varies with dose and pH [36]. For example, clinically relevant antibiotic classes (e.g. aminoglycosides and glycopeptides) are characterised by high solubility but demonstrate low permeability across hydrophobic biological barriers, while amoxicillin can have high predicted permeability but the solubility depends on the dose [36, 37]. Conversely, some poorly water-soluble antibiotics show good permeation across biological barriers (e.g. rifamycins and macrolides) [38]. At the site of action antibiotics need to permeate the bacterial cell envelope, which is particularly challenging for Gram-negative bacteria due to the presence of the outer lipopolysaccharide- and phospholipid- rich membrane and inner plasma membrane [39-41]. Resistance mechanisms that restrict the permeation of antibiotics through altered expression of porins and up-regulation of multidrug efflux pumps are furthermore a considerable challenge for all antibiotics [42, 43]. The restricted permeability into the bacterial cell envelope decreases the bioavailability of the antibiotic at the site of action and constitutes one of the largest challenges for the effectiveness of antimicrobials.

Depending on the site of administration drugs require different physicochemical properties to reach the intended site of action. Typically, the preferred route of administration for antibiotics is the oral route, due to its non-invasiveness, ease of administration and patient compliance. Oral drug delivery relies on the drug being soluble and permeable to cross the intestines and to be absorbed to cause an effect. Poorly permeable and poorly soluble drugs are challenging to develop as their properties typically lead to low absorption and decreased bioavailability following oral administration. Low bioavailability of antibiotics limits the attainment of therapeutically relevant drug concentrations at the site of infection compromising antimicrobial efficacy. While lower antibiotic plasma concentrations are required to clear acute infections with planktonic bacteria, biofilm-associated and chronic infections prevail in up to 80% of infectious diseases [44]. In this scenario, high and prolonged concentrations of antibiotics are required at the site of infection and are usually required to be dosed via intravenous administration.

Inadvertently, the systemic exposure to antibiotics from intravenous administration decreases the effective concentration at the site of infection compared to direct, local treatment. Moreover, systemic administration increases the development of resistance by exposing bacteria to sub-therapeutic levels of antibiotics [45]. Achieving supra-therapeutic concentrations of antibiotics via systemic administration is difficult due to broad, non-specific distribution and consequent increased risk of off-target side effects (e.g. cardiotoxicity, nephrotoxicity, ototoxicity, and peripheral neurotoxicity) [4]. The shortcoming of low

bioavailability from oral administration is not eliminated by intravenous administration and highlights the importance of local administration to the infection site.

Local delivery of antibiotics to the site of infection is an essential therapeutic consideration and could be the preferred option to achieve a spatially-confined high concentration of antibiotic, minimising unnecessary systemic exposure, reducing toxicity, and the development of resistance [4]. As infections primarily begin at a localised site, directed therapy to the site of infection will provide the greatest therapeutic benefit. However, multiple other factors need to be considered for the local delivery of antibiotics. For example, for adequate absorption via the pulmonary route of administration, the lung physiology, particle deposition, residence times, clearance mechanisms, and drug permeability all need to be taken into account [46]. For topical delivery, the therapeutic response is driven by the release of the drug from the dosage form, and penetration/diffusion across the upper and deeper skin layers [47]. This is co-dependent on the drug's physicochemical properties and also the dosage form or vehicle used. Importantly, further pharmaceutical development is required to produce effective local delivery systems for antibiotics, where currently, there are a limited number of antibiotics formulated and used for local administration.

3.2. Inability to overcome biological, chemical and physical barriers

The trend of high hydrophilicity or low partition coefficients generally results in low permeability of antibiotics through biological barriers, including across cell membranes needed to treat intracellular infections or epithelial tissue surfaces (i.e. skin, sinus, and pulmonary infections). The up-regulated host barriers, including inflammatory responses and mucus production, add further challenges to these biopharmaceutically challenged antimicrobial drugs. The dehydrated, thick mucus becomes a significant barrier for drug permeation across the epithelium, particularly in CF and other respiratory and gastrointestinal tract infections [48]. The EPS matrix is the first-line barrier in biofilms, where due to the overall negative charge of the EPS, cationic antibiotics (i.e. aminoglycosides and polymyxins) are bound to the outer matrix, rendering them highly ineffective against the bacterial community residing inside the biofilm (Figure 3) [49, 50]. At slightly less than neutral pH (pH 6-7) lipophilic and uncharged antibiotics (e.g. rifamycins and fluoroquinolones) can penetrate the EPS matrix, although changes in bacterial phenotype may alter the target sites of antimicrobial drugs [51]. The inhibition of bacterial cell division by fluoroquinolones has a limited effect on biofilm-based bacteria due to their switching to a slow growth rate [14, 52]. The unique interplay of genetic dominance and chemical barriers manifests as tolerance towards antimicrobial drugs that differ functionally and mechanistically from antimicrobial resistance [4, 17].

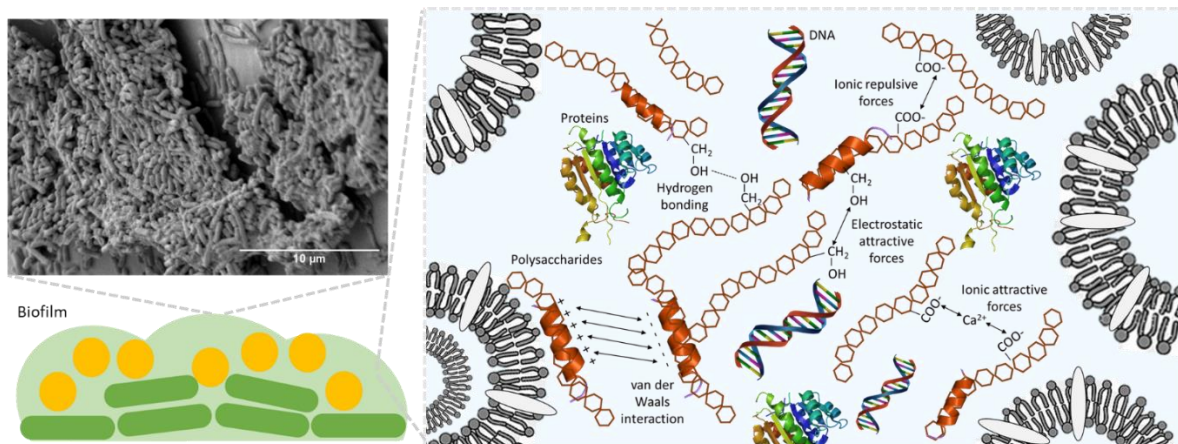


Figure 3. The chemical barriers of the EPS matrix of biofilms, as adapted from Flemming and Wingender [53], Copyright 2010, with permission from Springer Nature.

As schematically demonstrated in Figure 4, delivery of the antibiotic to the bacteria requires penetration across several biological, chemical and physical barriers, including the biofilm's EPS matrix, hydrophobic cell membranes in intracellular infections and host factors such as mucus and bodily secretions in the skin, eyes, noses, ears, or lung. Due to their physicochemical properties, achieving sufficient penetration may not be possible for antibiotics with high permeability coefficients without adequate formulation.

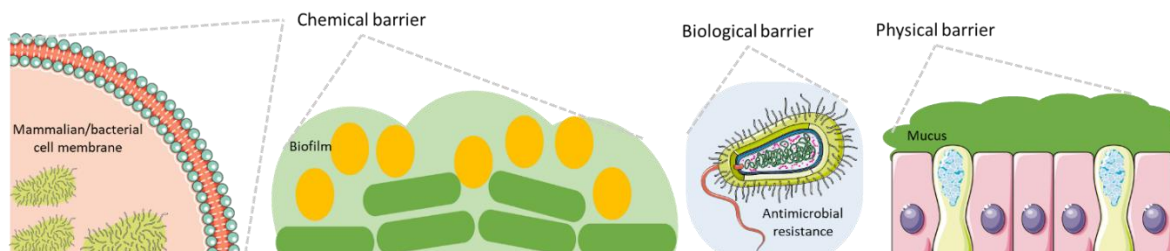


Figure 4. The hydrophobic cell membrane and EPS matrix of the biofilm are chemical barriers to antibiotics, while antimicrobial resistance mechanisms are biological barriers and the mucosal epithelia are physical barriers.

4. The structure and function of lipids as drug delivery systems

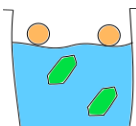
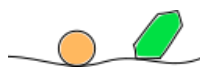
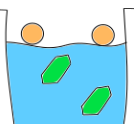
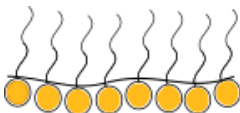
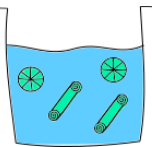
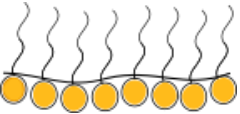
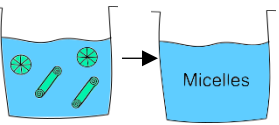
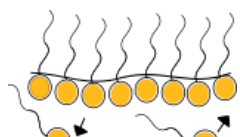
4.1. The physical chemistry of lipids

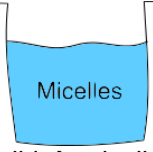
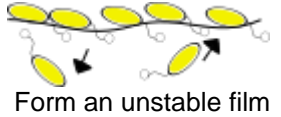
The structure and function of lipids are important in all biological systems and is primarily driven by the interaction between lipids within aqueous systems to form bilayers and other self-assembly structures. The assembly of amphiphilic lipids is driven by the hydrophobic effect, whereby hydrophilic solvents dissolve in similar solutes but repel hydrophobic solutes [54]. The unique structures formed are dictated to a less extent by the interplay of intermolecular forces between the amphiphiles [55]. This self-assembly phenomenon of lipids is vital to their biological functions, e.g. forming cellular membranes, storing energy, providing insulation, and transferring messaging between cells [56]. These properties also drive the function of lipids as soaps, detergents, emulsifiers, and surfactants. At the interplay

between chemistry and biology, lipids also serve as a critical ingredient in sanitation, food, cosmetic and pharmaceutical products.

In 1968, Small [57] classified biological lipids based on their interaction with water as either non-polar and polar. Non-polar lipids are long-chained alkanes or unsubstituted aromatic compounds either present as crystals or oils in water and do not spread as a monolayer on a surface. Polar lipids having at least one hydrophilic head group along the hydrocarbon chain can be split into three classes. Class I polar lipids are water-insoluble and non-swelling amphiphiles that remain as crystals or oils in a bulk aqueous environment and can spread at an aqueous surface to form a monolayer. Class II polar lipids are insoluble but can swell to form liquid crystals in water, due to the aliphatic chain becoming partly liquid at a specific temperature. Class III polar lipids are soluble amphiphiles that form unstable films at aqueous interfaces. Type IIIA soluble amphiphiles form liquid crystals in small volumes of water, before being solubilised. In contrast, Type IIIB soluble amphiphiles do not form liquid crystals (Table 1) [57].

Table 1. Classification of biological lipids in aqueous systems adapted from Small [57], Copyright 1968, with permission from John Wiley and Sons.

Class	Interaction with water		Examples
	Bulk	Surface	
Non-polar lipid	 Crystal or oil in water	 Will not spread to form a monolayer	Paraffins, large aromatic hydrocarbons
Polar lipids			
I. Insoluble non-swelling amphiphiles	 Crystal or oil in water	 Spreads to form a stable monolayer	Di-, triglycerides, long-chain protonated fatty acids, waxes, sterols
II. Insoluble swelling amphiphiles	 Liquid crystals (LC) in water	 Spreads to form a stable monolayer	Cell membrane lipids (i.e. lecithins), monoglycerides, acid soaps
III. Soluble amphiphiles			
A. Lyotropic mesomorphism	 Solid → LC → micelles	 Forms an unstable film	Anionic, cationic and non-ionic detergents

B. No lyotropic mesomorphism			Sulphated bile salts in fish and higher animals, steroids
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The geometric self-assembly of polar lipids in an aqueous system is driven by the critical packing parameter (CPP), which is a dimensionless ratio of the hydrophobic chain volume (V_s) to the product of the area of the hydrophilic head group (a_0) and the hydrophobic chain length (l) [58, 59].

$$CPP = \frac{V_s}{a_0 l}$$

When the CPP is less than $\frac{1}{3}$, spherical micelles form with a positive curvature of the lipid, and the hydrophilic headgroups face the outside (Class IIIb). If CPP is higher than $\frac{1}{3}$, but less than $\frac{1}{2}$, non-spherical, rod-like structures form, while at a $CPP > \frac{1}{2}$, planar aggregates form in a bilayer (Class IIIa). As the CPP increases (> 1), corresponding to the hydrophobic tail being more significant than the hydrophilic head group, inverted structures (e.g. inverse micelles) begin to form, including higher orders of liquid crystals (Class II) [60]. The CPP is an arbitrary classification and is also affected by environmental factors, including ionic strength, pH, water concentration, and temperature [61-64].

4.2. Lamellar and non-lamellar nanostructured liquid crystalline particles

The polar Class II and Class IIIa lipids that self-assemble into liquid crystals (LC) upon the addition of an aqueous solvent form various unique nanostructures, dependant on the CPP of the lipid [55]. As described by Kaasgaard and Drummond [65], the “ideal” amphiphile self-assembles with increasing concentration spans through micelles, micellar cubic, hexagonal, bicontinuous cubic, lamellar, inversed bicontinuous cubic, inversed hexagonal, inversed micellar cubic and inversed micelles mesophases. Figure 5A depicts the “normal” mesophases, and the varied shape of the amphiphile (including single carbon chained fatty acids and dual carbon chained diglycerides). The “normal”, lamellar and “inverse” assembly are represented in Figure 5B. These unique nanostructures are excellent drug delivery systems, where drugs can be loaded in the aqueous or lipophilic compartments according to the drug’s physicochemical properties.

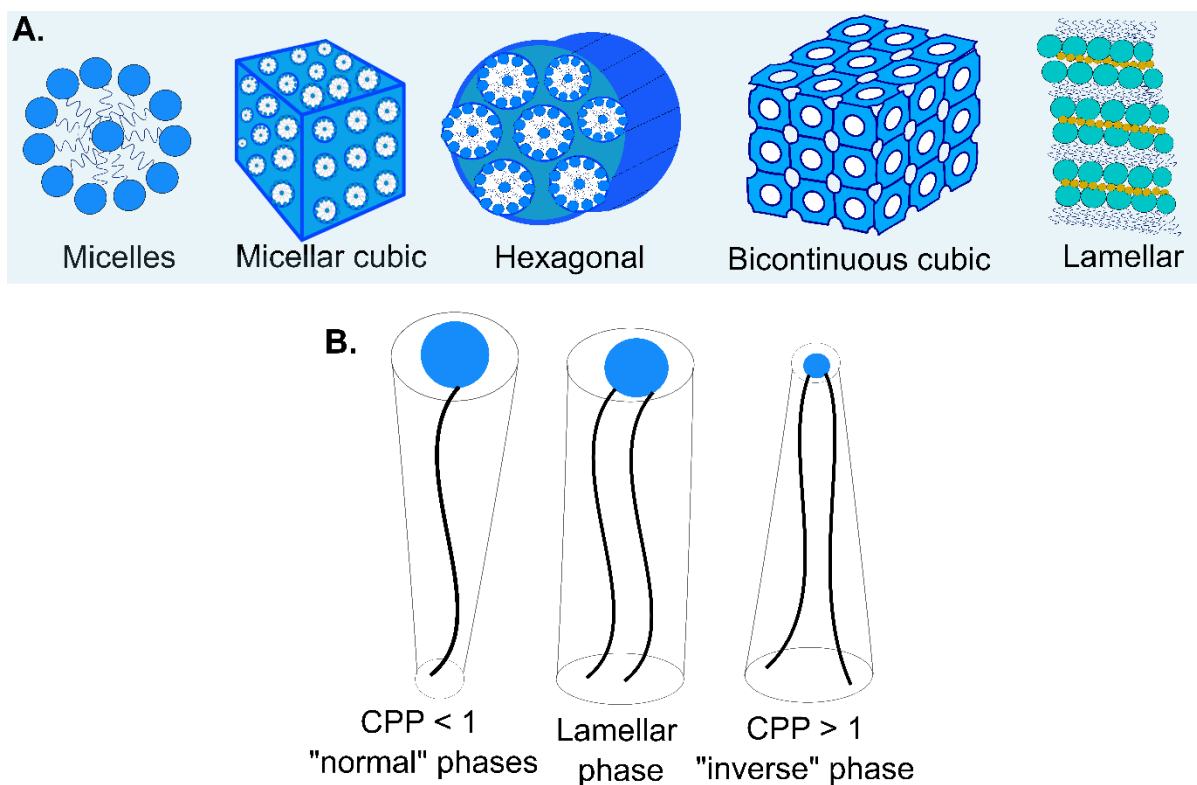


Figure 5A. Different phase structures of amphiphilic lipids and B. the specific structures of the amphiphile, adapted from Kaasgaard and Drummond [65], Copyright 2006, with permission from the Royal Society of Chemistry.

Positive curvature in the self-assembled lipid (i.e. $CPP < 1$) results in simpler nanostructures, including micelles up to lamellar phase structures. Dispersion of the lamellar phase creates one of the most extensively used drug delivery systems, liposomes [66]. The lipid bilayer shell of liposomes comprises phospholipid combinations that encase an inner aqueous cavity to load water-soluble drugs. Targeting ligands (e.g. proteins, antibodies, or peptides) or stabilising compounds (e.g. cholesterol, or polyethylene glycol) can be integrated into the lipid bilayer to improve the liposome function, in addition to loading hydrophobic or amphiphilic drugs. Drug loading is typically limited by rapid drug leakage from amphiphilic drugs partitioning in and out of the lipid membrane, as well as lower hydrophobic and hydrophilic interfacial regions, and limited solubilities [67].

Higher orders of the lyotropic lipid mesophases are non-lamellar and have a higher degree of negative curvature (i.e. $CPP > 1$). The higher CPP results in the formation of inverse nanostructures, beginning from the inversed bicontinuous cubic phase and beyond. For drug delivery, the (inverse bicontinuous) cubic and inverse hexagonal LC phases have received significant attention. These complex three-dimensional LC structures of the lipid have a higher interfacial surface area between aqueous domains and the ability to load considerable amounts of hydrophilic, hydrophobic and amphiphilic drugs [55, 61, 68]. Non-lamellar LC nanoparticles (hereafter denoted as LCNPs) are the LC mesophases dispersed as sub-micron particles and are often stabilised by synthetic block copolymers such as polyethylene glycol-polypropylene glycol-polyethylene glycol [68]. These LCNPs are also referred to as cubosomes and hexosomes, depending on their LC structure. Monoolein and phytantriol are two lipids primarily used to form the cubic or hexagonal phase LC structures, respectively. Obtaining the correct ratio and

concentration of these lipid and aqueous components is essential for specific LC formation, in addition to temperature, pH, and ionic strength.

The basic structural unit of liposomes and LCNPs are the LC lipid bilayers in different conformations and provide biomimetic features to both lipid nanoparticles. The complex structural geometry of the LCNPs offers advantages over liposomes, such as enhanced drug loads, increased stability, immediate- and sustained-drug release. Although, by far, liposomes have been more prevalent in innovating formulations for antimicrobials. For an extensive literature review on the use of liposomes in antimicrobial applications, the interested reader is referred to Forier *et al.* [6].

4.3. Solid lipid nanoparticles, nanostructured lipid carriers and lipid nanocapsules

The non-swellable polar Class I lipids (i.e. mono-, di-, triglycerides or fatty acids) that remain as crystals or oils in water are also useful drug delivery systems. Additional surfactants can stabilise non-soluble glycerolipids as solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs) and lipid nanocapsules (LNCs) [69]. These lipid-based nanoparticles are preferred systems for loading lipophilic drugs and provide extended stability during storage by dissolving the drug in an amorphous or molecularly dispersed state in the lipid core, reducing its exposure to the aqueous environment. Figure 6 schematically represents the physical differences between SLNs, NLCs and LNCs. SLNs contain only solid lipids (i.e. triglycerides, partial glycerides or saturated fatty acids) with rigid crystalline structures. In contrast, NLCs are a mixture of solid and liquid lipids (i.e. unsaturated fatty acids) with decreased crystallinity, which increases the drug loading capacity and release profiles [70]. LNCs are composed of a solid lipid core shielded by an oil or a lipophilic surfactant shell formed via a phase inversion temperature method.

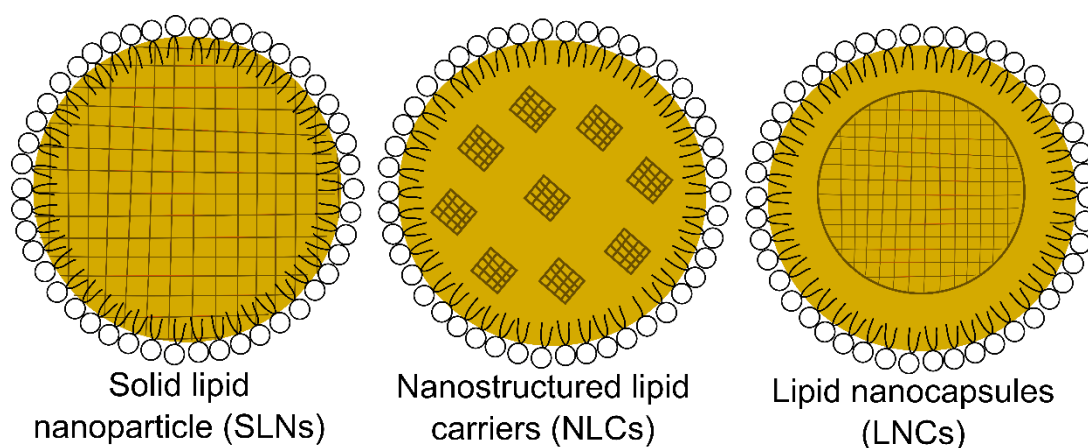


Figure 6. Schematic representation of solid lipid nanoparticles, nanostructured lipid carriers (NLCs) and lipid nanocapsules (LNCs).

When comparing the three systems, the crystalline structure of SLNs is less favourable than the disordered NLCs and LNCs with formless matrices allowing higher drug loading capacities [71]. The denser arrangement of lipids in these systems affords a greater lipophilic surface area to load bio-pharmaceutically challenging (low solubility and/or permeability) drugs, primarily through increasing the drug solubility [72-76]. Conversely, they are unable to load high amounts of water-soluble drugs and

require alternative methods, such as actively loading hydrophilic compounds onto surfactant capsules or utilising lipophilic pro-drugs, and hydrophobic ion-pairing [77, 78].

5. The mechanisms behind lipids and lipid-based drug delivery systems modulating the effects of antibiotics

The unique structural properties of lipids in aqueous environments drive their function in a biological environment and as drug delivery systems. The biological nature and amphiphilic properties of lipids render them excellent drug delivery systems to overcome the biological, chemical and physical barriers of bacteria and reduced permeability of antibiotics [79]. Lipid-based drug delivery systems (LBDDS) can increase antibiotics' permeability and availability at the site of infection, to obtain effective therapeutic antibiotic concentrations that lead to advance antimicrobial effects. This can reduce intravenous dosing requirements while maximising the antimicrobial effect, and minimising off-target toxicity, as reviewed elsewhere [6, 7, 80-83]. These benefits of LBDDS for antimicrobial therapy arise due to the unique properties and mechanisms of the lipids, including; (1) innate and synergistic antimicrobial activity, (2) fusion with the bacterial cell membrane, (3) nanoparticle characteristics that target bacteria and overcome barriers, and (4) triggered antibiotic release in the presence of bacteria.

5.1. Innate and synergistic antimicrobial effects

A seemingly forgotten function of lipids is their inherent antimicrobial properties. The antimicrobial properties of lipids were first recognised in the 1800s, advancing the scientific understanding of their use as soaps and disinfectants [84]. Their function as a soap relies on selective adsorption of the lipid to the surface or soil (e.g. grime, bacteria, fungi and viruses) to displace the soil from the surface under agitation. Later, lipids were discovered to play a crucial role in the function of the human innate immune system, showing potential as the first therapy for bacterial infections [85-87]. However, with the rise of antibiotics in the early 1900s, lipids were soon superseded as antimicrobial compounds.

The lipids that primarily demonstrate antimicrobial activity are fatty acids and monoglycerides. The bactericidal mechanism of action is non-specific and involves the insertion of the fatty acid carbon chain into the bacterial cell membrane. This disrupts and interferes with transmembrane signal transduction, or affects the electron transport chain and oxidative phosphorylation as summarised in Figure 7 [13]. Compared to a mammalian cell membrane, bacteria have a cell envelope composed of the cell membrane and cell wall. The cell wall contains peptidoglycan and differs between Gram-positive and Gram-negative bacteria, with a thicker peptidoglycan layer in Gram-positive bacteria. In contrast, Gram-negative bacteria have a second outer cell membrane containing more lipopolysaccharides. Thus, Gram-negative bacteria are less susceptible to the action of lipids than Gram-positive bacteria [13]. While overexpression of the peptidoglycan layer produces a thicker cell wall that could inhibit the insertion of the fatty acid, phenotypes resistant to fatty acids have not been observed, which is an attractive feature for clinical use [88].

The structure and shape of the fatty acid are pivotal to the function. Medium-chain fatty acids (6-12 carbons in chain length) demonstrate the most potent antimicrobial activity, where the minimum inhibitory concentration (MIC) decreases as the chain length of the fatty acid increases [88]. Specifically,

the free carboxyl group is deemed essential for antimicrobial activity, hence the availability of the functional group is crucial for the antibacterial effect. The type, position and degree of unsaturation also affect the antimicrobial action of the fatty acid, where one or two *cis* double bonds increases the antibacterial effect [88]. There are other differences in the dynamics of the antimicrobial effect, where monoglycerides containing medium-chain fatty acids have time-dependent antimicrobial properties compared to the respective fatty acids, which are concentration-dependent [89]. Sequentially, the variation in antimicrobial function is linked to the structure of individual lipid.

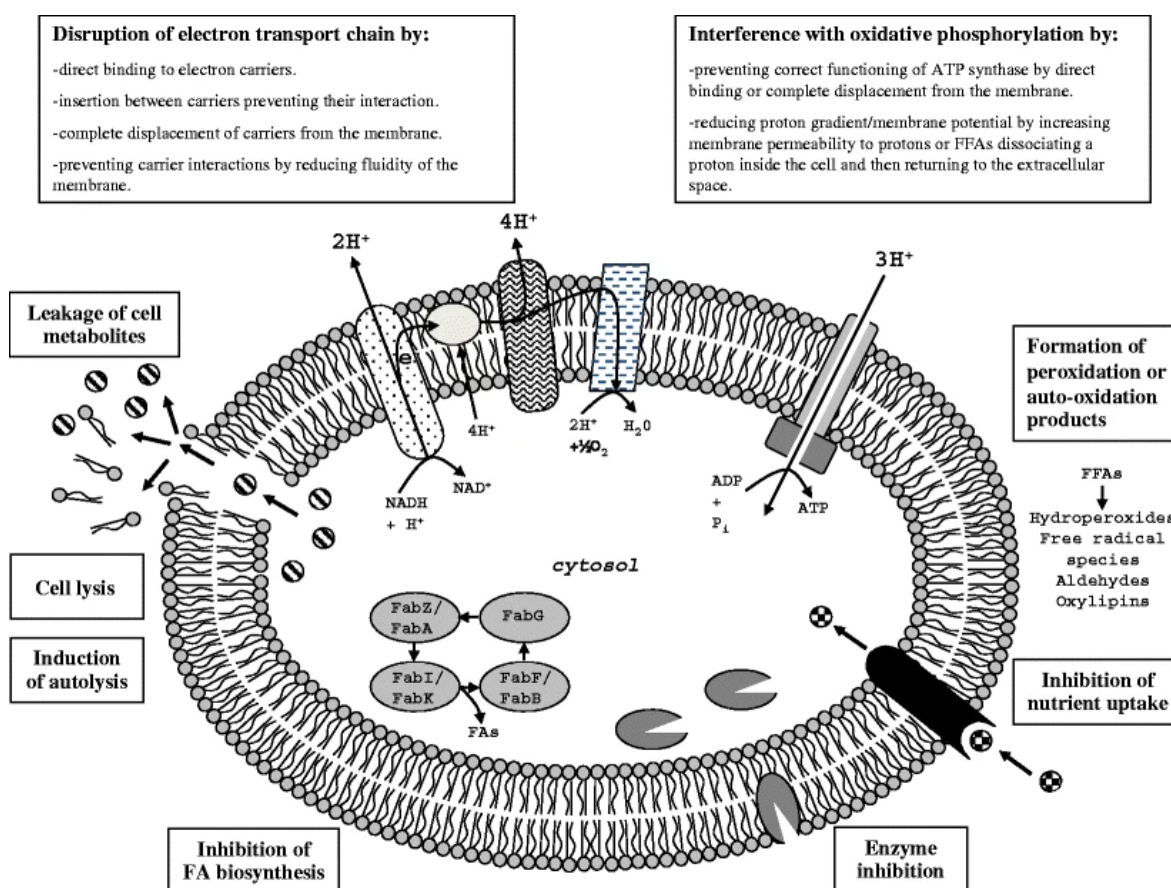


Figure 7. Proposed mechanism and possible cell targets of antimicrobial lipids as described and reprinted from Desbois and Smith [13], Copyright 2009, Springer Nature.

Monolaurin, the monoglyceride of the medium-chain fatty acid, lauric acid, is one of the most well-known antimicrobial lipids to date. It is marketed as a nutraceutical (Lauricidin[®]) by Med-Chem Laboratories Inc. and as a pharmaceutical product by Hennepin Life Science [88, 90]. As monoglycerides are poorly water-soluble, drug delivery systems are required to improve their solubility, activity and utility during pharmaceutical development. Currently, a non-aqueous gel composed of propylene glycol, polyethylene glycol, and hydroxypropyl cellulose is under development [91]. In New Zealand white rabbits with a surgical wound infected with *S. aureus* (1×10^9 colony forming units (CFU)/mL), the 5% monolaurin gel completely eradicated the infection within 24 hours. After 1 hour, the 5% monolaurin gel also eliminated 3-log and 7-log of *P. aeruginosa* and *Acinetobacter baumannii*, respectively from the surgical wounds [91]. Currently, phase II clinical trials for monolaurin have begun for bacterial vaginosis

and candidiasis. At the same time, preclinical testing has been completed for the urinary tract, skin and soft tissue infections, demonstrating advantages in the clinical environment [92].

5.1.1. Innate antimicrobial systems

Glycerolipids are a key ingredient in most LBDDS. In SLNs, NLCs and LNCs, glycerolipids are the backbone of the lipid nanoparticle's structure. Using antimicrobial-active glycerolipids can form innate antimicrobial SLNs, NLCs and LNCs in the absence of a loaded antibiotic. Umerska *et al.* [89] investigated a range of LNCs containing various fatty acids and monoglycerides. By substituting lecithin, a commonly used co-surfactant to form LNCs, with active antimicrobial monoglycerides and fatty acids, the LNCs had built-in antimicrobial properties. LNCs (without antibiotics) composed of caproic (C₆), caprylic (C₈), capric (C₁₀) and lauric acid- (C₁₂) had a minimum inhibitory concentration (MIC) for *S. aureus* of 1.88, 0.938, 0.470 and 0.234 mg/mL, respectively. The antimicrobial performance of the LNCs corresponded to the properties of the included fatty acid, which was more potent against Gram-positive bacteria and increased with the carbon-chain length of the fatty acid [13]. Correspondingly, when formed with monoglycerides, the antimicrobial effect of LNCs further increased against *S. aureus* [89]. As depicted in Table 2, solubilisation of the fatty acid or monoglyceride in LBDDSs further increased the antimicrobial activity compared to application in an organic solvent (i.e. dimethyl sulfoxide [DMSO]). The elimination of the organic solvent further extends the potential clinical application of the fatty acids and monoglycerides as antimicrobial therapies.

Table 2. Minimum inhibitory concentration (MIC) of various fatty acids and monoglycerides against *S. aureus* from American Type Culture Collection (ATCC), NI = not inhibitory and NA = not assessed

Glycerolipid	Chain length	Formulation	MIC <i>S. aureus</i> (mg/mL)	Haemolytic concentration (mg/mL)	Ref
Caproic acid	C6, saturated	LNCs	1.9	0.9	[89]
		DMSO soln	NI	-	
Caprylic acid	C8, saturated	LNCs	0.94	0.45	[89]
		DMSO soln	NI	-	
Capric acid	C10, saturated	LNCs	0.45	0.45	[89]
		DMSO soln.	0.50	-	[88]
Lauric acid	C12, saturated	LNCs	0.23	0.45	[89]
		DMSO soln.	0.49		[88]
Monocaprin	C10	LNCs	0.12-0.23	0.45	[89]
Capmul MCM	C8, C10	LNCs	0.08	-	[93]
Monolaurin	C12	LNCs	0.015	1.8	[89]
		Non aqueous gel	0.5	-	[91]
Myristic acid	C14, saturated	DMSO soln.	1.0	NA	[88]
Linolenic acid	C18, 3 unsaturated	Liposomes	0.5	NA	[88, 94, 95]
		DMSO soln	0.50	-	
Oleic acid	C18,	Liposomes	0.010 (MRSA)	NA	[96]

1 unsaturated	DMSO soln	NI	-
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While fatty acids and monoglycerides imparted antimicrobial properties on the LNCs, in some cases, this also increased the toxicity of the lipid nanoparticles against healthy cells. Along with the ability to interfere and destabilise the bacterial cell membrane, lipids can destabilise eukaryotic cell membranes, causing cell lysis. While lipids are generally recognised as safe (GRAS) materials, monoglycerides and fatty acids have varying toxicity profiles, depending on concentration, cell line and conditions employed. The potential of lipids to destabilise membranes is more pronounced when in direct contact with a single cell monolayer compared to topical application to tissue in an animal model [97]. LNCs containing capric, caprylic, and caproic acids were haemolytic in horse erythrocytes at the same concentration that inhibited bacterial growth (Table 2) [89]. In this scenario, the advantage of the antimicrobial effect is outweighed by the toxicity of the LNCs. In comparison, monolaurin LNCs were non-toxic at their antimicrobial concentrations enabling use as both an antimicrobial agent and as an excipient for the design of antimicrobial drug delivery system. All of which further demonstrates the strong clinical potential of monolaurin LBDDS to advance antimicrobial therapies.

Other LBDDS that are formed from glycerolipids, including LCNPs, have yet to demonstrate innate antimicrobial activities. Unlike other monoglycerides (e.g. monolaurin), the most common lipid used to form LCNPs, monoolein has not demonstrated antimicrobial activity at non-toxic concentrations, which is likely due to its long, saturated hydrocarbon chain. However, the aliphatic alcohol, phytantriol also commonly used to form LCNPs, is patented as an antimicrobial preservative in cosmetic preparations, [98], yet concentration is imperative to effect. Other fatty acid and monoglyceride combinations can form LCNPs which may be strategically designed to be intrinsically active, including a monolaurin LC system [99]. Similarly, innate antimicrobial activity has been previously achieved with liposomes doped with oleic and linolenic acid [94-96]. Harnessing the innate antimicrobial activity of specific lipids can be very advantageous to build smart, innately active LBDDS.

5.1.2. Synergistic antimicrobial systems

“Two is better than one” is an analogy that has not always been accepted in basic antimicrobial therapy guidelines. However, with the rise in antimicrobial resistance, targeting two or more functions of the bacteria has proven benefits in reducing further development of resistance [100]. For example, combination therapies are necessary for tuberculosis and *Helicobacter pylori* infections [101]. An additive effect is defined as the sum of two or more compound’s effects, whereas synergy is greater than the effect of the two compounds added together [102]. Clinically, a synergistic antimicrobial effect is most beneficial when using two compounds with different modes of action. The significant advantage of this phenomena is the ability to reduce the dosage requirement of the antibiotic which would have otherwise not been achievable due to toxicity concerns; this is explicitly relevant in biofilm infections where up to 1000-fold higher concentrations of antibiotics may be required for efficacy.

Glycerolipids are commercially available in pharmaceutical grades from various sources (e.g. Capmul MCM and Imwitor) and frequently used for multiple purposes in a variety of formulations [93]. These

commercial lipids have demonstrated synergistic effects with antibiotics, by increasing their potency against bacteria in both planktonic and biofilm states. For example, the susceptibility of gentamicin to clinical isolates of *S. aureus* increased by 10-fold in the planktonic state by the addition of 0.25 mg/mL Capmul MCM, and 320-fold in the biofilm state by the addition of 1 mg/mL Capmul MCM. The antibiotic classes that benefited from the glycerolipids included; aminoglycosides, carbapenems, cephalosporins, glycopeptides, macrolides, phenicols, and quinolones. The increased potency occurs across important clinically relevant bacteria, including; *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*, otherwise known as ESKAPE pathogens [93]. As these glycerolipids are frequently used in a variety of LBDDS, exploiting the synergistic antimicrobial combination has clear advantages in clinical development.

While the mechanism behind the antimicrobial effect of fatty acids and monoglycerides is non-specific [13], the specific molecular mechanism behind the synergistic effect between each glycerolipid and antibiotics combination is not entirely understood. Synergistic effects have also been described with monolaurin LNCs and antimicrobial peptides, including the plectasin derivatives AP114 and AP138. The monolaurin LNCs synergistically increased the antimicrobial effect against methicillin-resistant *S. aureus*, decreasing the effective concentration of the two antimicrobial peptides [12]. However, contrary to *in vitro* results, in a murine incision wound model, the antimicrobial performance of the antimicrobial peptide DPK-060 did not differ between monolaurin-LNCs and lecithin LNCs [103]. The discrepancies between *in vitro* and *in vivo* results highlights the need for rigorous characterisation and understanding of the molecular basis behind the synergistic effects occurring, which may drive more fruitful clinical development.

Furthermore, the non-specific antimicrobial effect of glycerolipids has primarily been investigated in the planktonic state, whereas little is reported on the effect in biofilms. Moreover, as fatty acids are generally more active against Gram-positive bacteria, they are rarely further investigated in Gram-negative bacteria. Conversely, linolenic acid is a fatty acid that can disrupt quorum sensing in *P. aeruginosa*, inhibiting biofilm formation. Combining the action of linolenic acid and tobramycin has been a beneficial strategy to improve the total antimicrobial effect in *P. aeruginosa* biofilms. The disruption of the *P. aeruginosa* biofilm communication by linolenic acid facilitated the inhibition of bacterial protein synthesis by tobramycin and helped to eradicate *P. aeruginosa* biofilms [94]. The linolenic acid and tobramycin combination showcases the advantages of understanding the molecular mechanisms and combining multiple antimicrobial targets in *P. aeruginosa* biofilms. Combinations of glyceryl palmitostearate, glyceryl behenate, and tristearin in SLNs have shown a 7-fold increase in the anti-virulence activity of pyocyanin quorum-sensing inhibitors [104]. This effect was further modulated by the surfactants poloxamer 407 and polysorbate 80 used for the preparation of SLNs, inhibiting pyocyanin without the quorum-sensing inhibitors by an unknown molecular function. Together, the synergistic effects of glycerolipids based LBDDS can be highly beneficial for antimicrobial therapies, and rigorous characterisation and evaluation is warranted for the future development of smart, synergistic systems.

5.2. Biomimetic properties: fusion with the bacterial cell membranes

The quintessential biological properties of lipids are an unequivocal benefit of LBDDS. In a biological environment under conditions such as acidic pH or the presence of cations, lipids can undergo a phase transition that facilitates their interaction with cell membranes, promoting reciprocal mixing and membrane destabilisation. Cell membranes are composed of phospholipids as a lamellar bilayer and supplemented with cholesterol and various (lipo)-proteins. Liposomes drive increased cellular uptake of drugs through fusing with cell membranes, which results in the release of the cargo inside the cell's cytoplasm [105]. This form of directed and targeted therapy is a widely known advantage of liposomal systems.

Liposomes are known to interact with the cell membrane and increase the intracellular uptake of drugs. There are four different types of interactions between liposomes and cell membranes that enable this, including; (1) adsorption, (2) endocytosis clathrin-(in)-dependent, (3) lipid exchange and transfer across the cell membrane and (4) fusion with intracellular membranes [106-108]. Enhanced uptake of antibiotics has been observed following the fusion of mammalian cells and liposomes based on 2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC), and 1,2-dimyristoyl-rac-glycero-3-methylpolyoxyethylene (DMG-PEG₂₀₀₀) and penicillin G conjugated to the phospholipid chain. As a result of enhanced endocytosis of the liposomes, the uptake of penicillin G increased by 8-10% and this translated to a significantly improved antimicrobial effect against an *S. aureus* infected alveolar epithelial cell line (A549), eliminating 99.9998% of the bacteria [109]. In intracellular infections, liposomes are widely regarded as advantageous to deliver antibiotics across the hydrophobic cell membrane and other host-related barriers, as previously described [110, 111].

The bacterial cell membrane is similar in composition to the mammalian cell membrane, but a peptidoglycan-based cell wall surrounds it, forming the cell envelope. For Gram-negative bacteria, the additional outer membrane comprises phospholipids and lipopolysaccharides. Despite the concealed cell membrane, lipids destabilise the bacteria membrane via insertion of their hydrocarbon chain into the bacterial cell membrane to produce the bactericidal effect [13]. Similarly, liposome fusion with the cell membrane can occur with reciprocal mixing of lipid-based membranes [112].

Fluidosomes™ were conceived on the discovery that liposomes with decreased membrane fluidity undergo fusion with bacterial cells, potentiating the effect of antibiotics. Fluidosomes™ are negatively charged liposomes composed of dipalmitoylphosphatidylcholine (DPPC) and dimyristoyl phosphatidylglycerol (DMPG) [113]. In Sprague-Dawley rats with a respiratory tract infection, tobramycin Fluidosomes™ eradicated all *P. aeruginosa* (inoculum: 1×10^6 CFU/mL). *P. aeruginosa* eradication was not achieved with tobramycin in uncharged di-stearoyl phosphatidylcholine (DSPC) and dimyristoyl phosphatidylcholine (DMPC) liposomes or as an unformulated solution [114]. The DPPC: DMPG Fluidosomes™ displayed a lower degree of crystallinity and rigidity compared to DSPC: DMPC liposomes; where this increased fluidity, promoted fusion with the outer bacterial cell membrane of Gram-negative *P. aeruginosa* and increased the concentration of tobramycin in the bacterial cytoplasm (*Figure 8A*) [112, 114, 115]. The Fluidosome™ technology also enhanced the concentration of tobramycin in the lung following pulmonary administration, unlike unformulated tobramycin. The

Fluidosome™ tobramycin formulation was granted orphan drug status in Europe in 2006 and was licensed to Axentis Pharma in 2008, although since 2013, the sponsor withdrew this status [116]. Analogous liposomes are under development to enhance the anchoring and fusion to bacterial cell membranes by the inclusion of fatty acids such as lauric, oleic, and linolenic acid into the lipid bilayer [79, 95, 96]. The targeted antibiotic delivery by bacterial membrane fusion is an enormous benefit of liposomes that is driving clinical development.

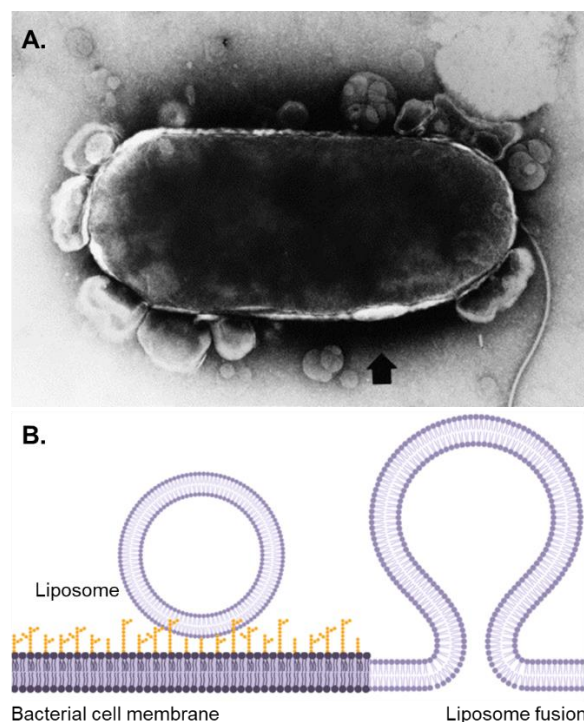


Figure 8. A. Interaction of Fluidosomes™ with *Pseudomonas aeruginosa* 429 cells as observed by negative staining with phosphotungstic acid. Magnification: $\times 50\,561$, reprinted from Sachetelli *et al.* [112], Copyright 2000, with permission from Elsevier. B. Schematic of liposomes fusing to the bacterial membrane.

In comparison to the extensive body of literature on liposomes to enhance the antimicrobial effect, less work has been reported on LCNPs. Cubic phase LCNPs (i.e. cubosomes) have bilayers organised in space based on infinite periodic minimal surfaces providing a highly tortuous structure and greater surface area for interaction, which provides fusogenic properties similar to liposomes and increases cellular uptake [117-119]. Cubosomes have emerged as fusogenic nanoparticles to facilitate higher drug uptake into mammalian cells with the cubic LC phase as the potential intermediate in the membrane fusion mechanism [120-122]. While phytantriol-based LCNPs have demonstrated enhanced fusion with membranes compared to monoolein-based LCNPs, the former have shown increased haemolysis of healthy cells and propensity to disrupt the cell membrane [118, 123]. LCNPs have also demonstrated superior skin retention compared to liposomes due to the greater surface area of the lipid bilayer, facilitating interaction with the hydrophobic dermis layer of the skin [124].

Boge *et al.* [125] visualised the interaction of LCNPs loaded with antimicrobial peptide (LL-37) and Gram-negative bacteria using super-resolution laser scanning microscopy and cryogenic electron tomography. Furthermore, greater adsorption of the LCNPs loaded with LL-37 into the supported lipid

bilayer was identified by a larger drop in frequency and a corresponding increase in dissipation via QCM-D (quartz crystal microbalance with dissipation) monitoring compared to LL-37 and LCNPs individually. Neutron reflectivity further demonstrated direct interaction of LCNPs loaded with LL-37 with the lipid bilayer membrane of *E. coli* cells with a Bragg peak occurrence, which did not occur for LL-37 alone, suggesting the LCNPs act as an adjuvant-like antimicrobial carrier for LL-37 ([125]). In Figure 9B, the scattering density length profiles show a more dense lipid bilayer when LCNPs loaded with LL-37 interacted with a lipid bilayer membrane compared to unloaded LL-37 and LCNPs alone. This was indicative of the LCNPs and the antimicrobial peptide acting as an ‘antimicrobial unit’ against *E. coli*.

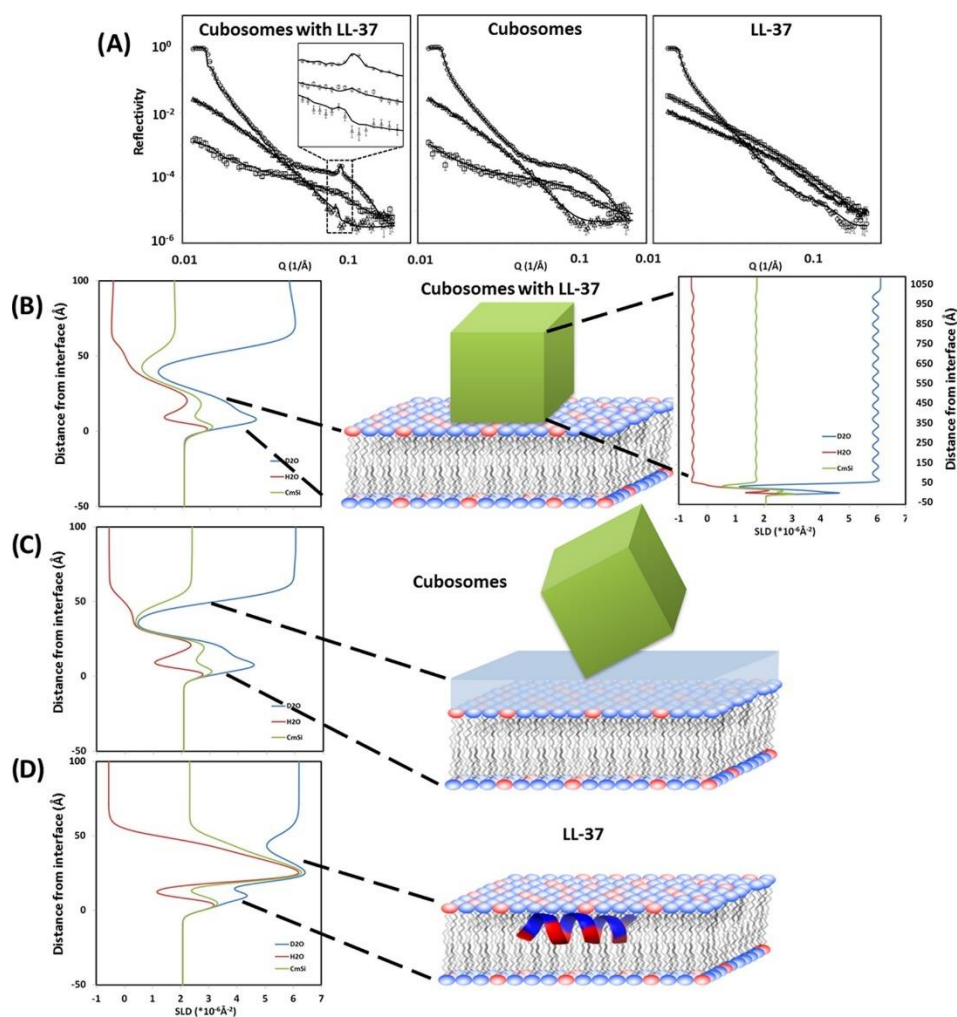


Figure 9. A. experimental (markers) and fitted (solid lines) NR profiles of dDMPC/dDMPG bilayers after exposure to cubosomes with LL-37 (the inset shows magnification around the Bragg peak), cubosomes without LL-37, and pure LL-37, experimental data were collected in three contrasts: d-buffer (circles), Cmsi-buffer (squares), and h-buffer (triangles). Scattering length density profiles and schematical models of the bilayers are presented in (B)–(D). The cubosomes in illustrations (B) and (C) are not to scale. Reprinted with permission from Boge et al. [118]. Copyright, 2019, American Chemical Society.

The extended benefits of the antimicrobial peptide and LCNPs acting ‘antimicrobial unit’ remains relatively unknown. Perhaps the unit can alter the antimicrobial’s structure and therefore, the bacteria’s recognition of antibiotics, which could overcome antimicrobial resistance mechanisms. Presently there is no clear evidence of LCNPs enhancing the efficacy of antibiotics. In planktonic *S. aureus*, LCNPs

slightly decreased the MIC of rifampicin from 0.1 µg/mL to 0.05 µg/mL [126]. However, as the clinical breakpoint for rifampicin against *S. aureus* is 0.1 µg/mL, this effect was not clinically significant. LCNPs are underexplored in terms of the benefits they could provide antimicrobial therapies in overcoming the barriers of bacteria. Nevertheless, harnessing the potential of LCNPs to interact with the bacteria should guide future evaluation and characterisation as an effective LBDDS for antimicrobial therapies.

5.3. Nanoparticle characteristics that target bacteria and overcome barriers

Nanoparticulate based-LBDDS harbour functional characteristics that seemingly go unrecognised in their effect on potentiating the effects of antimicrobial compounds. These include the nanoparticle size and surface charge and useful surface stabilisers that enables higher permeation through the bacterial and host barriers. Specifically, electrostatic interactions between the LBDDS and bacteria can direct drug delivery systems to a target site of infection with highly specific attractive forces [127]. Strategic selection of surface stabilisers permits penetration across physical and chemical barriers such as mucus or the EPS matrix of biofilms [128]. Fine control over the particle size and surface properties of drug delivery systems is a feature that broadly applies to other carriers, including polymeric or inorganic drug delivery systems [80]. However, unlike these other carriers, the particle size and surface characteristics of liposomes and LCNPs are complementary to their biomimetic features. Similarly, the size and surface properties of SLNs, NLCs and LNCs are complimentary with the inherent antimicrobial properties of the lipids. Table 3 highlights the different attractive features of LBDDS that are discussed in the following sections.

Table 3. Notable LBDDS that demonstrate greater biofilm penetration or targeting of bacteria

Effect	Attractive surface characteristic	LBDDS	Composition	Ref
Increased penetration & anti-biofilm effect	Low negative surface charge / membrane fluidity	Liposomes	DPPC: DMPG	[112, 114, 129]
Increased penetration & anti-biofilm effect	Neutral surface charge/300 nm particle size	Liposomes	DPPC: cholesterol	[130].
Biofilm targeting	+60 mV surface charge / 130 nm particle size	Liposomes	DSCP:DOTAP	[131]
Increased penetration	-22 mV surface charge / 250 nm particle size	Liposomes	DOPC:DPPG	[132]
Bacterial targeting & increased anti-bacterial (planktonic) effect	+30 mV surface charge / 135 nm particle size	LCNPs	DOTAP-monoolein	[126]

Increased penetration in artificial mucus	Poloxamer 407/polysorbate 80 surface coating Slightly negative surface charge / 50-150 nm particle size	SLNs	Glyceryl palmitostearate, glyceryl behenate and tristearin with either poloxamer 407 or polysorbate 80	[104, 133]
Increased mucus penetration and anti-biofilm formation	Polysorbate 80 surface coating suggesting mucus penetration -20 mV surface charge / 180 nm particle size	LNCs	Poly(ϵ -caprolactone), sorbitan monostearate and oleic acid	[134]

5.3.1. Using electrostatic interactions for targeting bacteria

The cell envelope of bacteria generally imparts a negative charge, prominently from the teichoic acids attached to the cell membrane in Gram-positive cells and the lipopolysaccharides in Gram-negative cells [135, 136]. For this reason, cationic drugs or delivery systems are usually employed to target bacteria [136]. For example, antimicrobial peptides, with a positive charge, are heralded as a new generation of targeted antibiotics that attract and insert into the bacterial cell envelope with additional immunomodulating effects [137]. However, while bacteria are negatively charged, the environment surrounding the bacteria can also contribute and affect these electrostatic interactions occurring during the targeted antibiotic delivery. When bacteria cluster into biofilms, the extracellular DNA and various polysaccharides in the EPS matrix including alginate, impart an additional overall negative charge [138]. In this scenario, cationic antibiotics (e.g. aminoglycosides and colistin sulphate) bind to the negatively charged EPS matrix, immobilising the antibiotic and limiting access to the encased bacteria [139]. The negative charges within the EPS matrix hinder the effects of antibiotics and drug delivery systems, increasing the complexity of using simple electrostatic interactions for targeting bacteria.

The effect of cationic drug delivery systems interacting with the EPS matrix is multifarious. Liposomes have been a prominent formulation approach for various aminoglycoside antibiotics in biofilm-related infections. The lipid bilayer core provides an excellent shield to conceal the cationic antibiotics in the aqueous core, enabling higher permeation across the EPS matrix of biofilms. In addition to fusion with the bacterial cell membrane, as previously discussed [140]. Employing different lipid combinations enables tailoring of the surface charge and size of liposomes, where multiple combinations have been examined and widely reviewed by Forier *et al.* [6]. Interestingly, both positively- and negatively-charged liposomal systems have been trialled, each with a unique targeting argument.

Cationic, uni-lamellar liposomes have demonstrated higher innate antimicrobial activity compared to 1000 nm multilamellar vesicles/ liposomes, due to the combined effects of electrostatic attraction and

small particle size (~150 nm) [131]. However, in a different study, larger (~ 250 nm) cationic (+30 mV) liposomes were immobilised in the EPS matrix, thereby not reaching the encased bacteria and relied on the loaded antibiotic to reach the inner bacterial community. In comparison, negatively charged (-22 mV) liposomes of similar size were more likely to penetrate the biofilms EPS matrix and were detected closer to the bacterial cells [132]. Interestingly, the anionic liposomes did not potentiate the antimicrobial effect of tobramycin compared to free drug, which may be because of electrostatic repulsion from the negatively charged bacterial cell membrane and low level of antibiotic loading.

As a consequence of non-ionic surfactants being used to stabilise LCNPs, the particles generally have a slightly negative or near-neutral surface charge, which can also be controlled by loading different antibiotics or other additives into the structures [141]. The addition of cationic dioleoyl-3-trimethylammonium propane (DOTAP) to rifampicin loaded monoolein-LCNPs decreased the MIC against planktonic *S. aureus* from 0.1 µg/mL to 0.03 µg/mL [126]. The electrostatic interaction permitted more co-localisation events between the cationic nanoparticles and negatively charged *S. aureus*. Reductions in fluorescence resonance energy transfer (FRET) confirmed that the cationic nanoparticles fused with the bacteria cells, which facilitated the enhanced antimicrobial effect [126]. In this case, electrostatic interactions lead to an enhanced fusion of the nanoparticle with bacterial cell membranes. Comparatively, the interaction between the bacterial cells and the unmodified monoolein LCNPs was not explored. Moreover, understanding the specific electrostatic targeting modalities in the clinical situation, where bacteria primarily survive in biofilms rather than single colonies, is of extreme importance. In the case of biofilm infections, the positive charge may adversely affect the action of LCNPs, as aforementioned with the liposomes [132] and is yet to be examined.

While a sub-micron particle size is desirable, there is little consensus on what surface charge best targets biofilm infections. Seemingly, either highly positive or negative charges tend to increase restrictions on the nanoparticle reaching the site of bacteria. Moreover, cationic nanoparticles are typically more toxic than anionic particles due to their strong interaction with the negatively charged mammalian cell membrane. However, different cell types may internalise different particle morphologies to different degrees. In general, non-phagocytic cells preferentially take up cationic particles [142]. In contrast, anionic particles are more likely to be phagocytosed by macrophages due to resembling negatively charged bacteria cells [143]. Correspondingly, anionic liposomes may be more beneficial for intracellular infections that reside inside phagocytic cell types. For example, a 3-fold increase in the uptake of rifampicin in the lungs of albino rats resulted from rifampicin loaded anionic liposomes compared to the free antibiotic and neutral liposomes [144, 145]. Undoubtedly, every nanoparticulate system has its own advantages and disadvantages.

In (commercially) successful liposomal systems, the negatively-charged DMPG in Fluidosomes™ was fundamental in providing decreased fluidity and bacterial cell membrane fusion [112]. While in comparison, a neutral surface charge of the sub-micron DPPC and cholesterol liposomes was essential to produce the enhanced antimicrobial effect of amikacin, which was not achievable with a highly negatively charged liposomes (i.e. -25 to -40 mV) [130]. The amikacin liposomal formulation has later been the first commercialised liposomal antibiotic product, called Arikayce®.

Overall, for targeting bacteria in the clinical environment, understanding the surrounding environment is crucial to the design of the targeting modality. For intracellular infections, specific cellular uptake is essential and can be guided by intricate electrostatic interactions, such as macrophages recognising foreign anionic particles. In comparison for biofilm targeting, cationic lipid nanoparticles can stick to the EPS matrix as a strategy that requires the antibiotic to do the rest of the work. While to overcome the EPS matrix, a slightly negative or near-neutral charged lipid nanoparticle is proposed to facilitate greater antibiotic penetration and effect.

5.3.2. Surface functionalisation for mucus penetration to reach bacterial communities

The upregulated inflammatory response and increased mucus production in response to bacterial infections result in significant biological and physical barriers to antimicrobial therapies [146]. Mucus is a viscous substance primarily composed of mucins which sterically hinder the diffusion of many compounds and delivery systems [133]. Mucus-penetrating particles reduce the steric hindrance experienced and can diffuse through mucus. These particles most commonly have a diameter less than 300 nm and coated with polyethylene glycol (PEG) can diffuse through the pores of mucus, regardless of the composition of the inner core [128, 147]. Contrary to mucus penetration are mucus adhering particles, which do not penetrate but rather attach to the mucus, decreasing particulate removal following mucociliary clearance [148], as schematically represented in Figure 10. While mucus-adhering particles were considered to be more beneficial, mucus-penetrating particles are now considered to provide greater therapeutic effects.

Pluronic® F-127/poloxamer 407 is a commercially available triblock copolymer of PEG-polypropylene glycol-PEG that has been used as a biocompatible surface coating of PLGA nanoparticles to form mucus penetrating particles [149]. These mucus penetrating particles can reach the underlining epithelium coated with native CF sputum in the trachea following pulmonary administration to mice. In contrast, uncoated PLGA nanoparticles remained on top of the mucus [149].

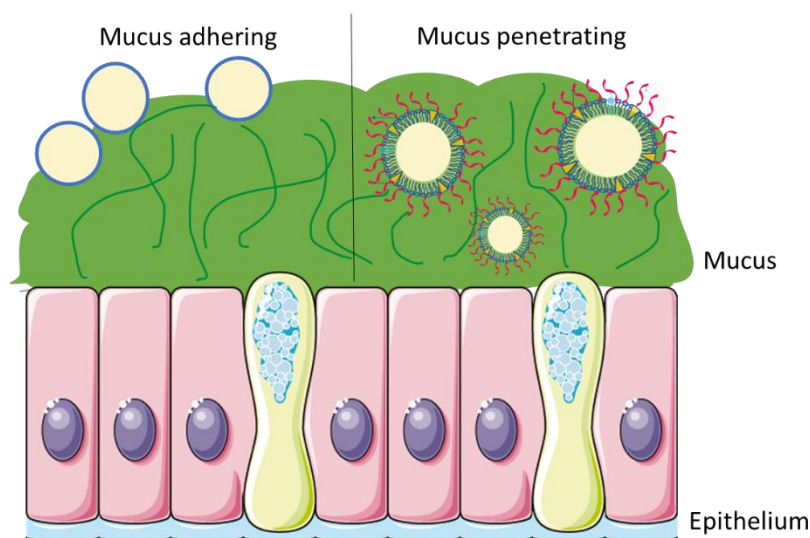


Figure 10. Comparison between mucus adhering and mucus penetrating nanoparticles

Pluronic® F-127 is usually the surfactant of choice to stabilise SLNs and LCNPs [150, 151]. The mucus-penetrating properties of SLNs are dependent on the surfactant used; SLNs possessing a core prepared from glyceryl palmitostearate (Precirol®), glyceryl behenate (Compritol®) and tristearin, stabilised using either poloxamer 407 or polysorbate 80 as the surfactant, were more likely to penetrate across artificial sputum mucus compared to polyvinyl alcohol coated SLNs [104, 133]. Chemically, artificial sputum mucus (ASM) mimics the composition of pulmonary mucus, particularly the concentration of mucins, which act as a significant chemical barrier in native mucus [152]. However, differences arise in the mechanical and rheological properties between ASM and native mucus, where ASM has a considerably lower viscosity and viscoelasticity [153]. In a later study, the same group found discrepancies in the diffusion of SLNs through ASM, CF sputum and a Calu-3 cell line producing mucus. The diffusion of the SLNs stabilised using polysorbate 80 through CF sputum was considerably restricted, while the poloxamer 407 or Pluronic® F-127 stabilisers led to greater mucus-penetration [151]. While mucus surrogates are economical and plentiful, it is imperative that *in vitro* models resemble the *in vivo* environment as closely as possible. For this reason, ASM can be modified with gel-forming polymers such as polyacrylic acid to form more bio-relevant viscoelastic models of mucus [153]. Furthermore, it is not yet evident whether enhanced penetration through mucus will lead to an improved antimicrobial effect. There are limitations to the studies investigating mucus-penetrating SLNs for antibiotics, where most do not include mucus in the bacterial infection model [104, 134, 154], highlighting the importance of understanding the models used and correlating the effect to the physiological and clinically relevant environment.

In contrast, LC phases have been described with mucoadhesive properties [64, 155-157]. The cubic phase structure was essential for mucoadhesion of monoolein LCs within porcine's stomach, intestine and buccal mucosa [155], although phytantriol and oleic acid LCNPs with hexagonal phase structure demonstrated greater mucoadhesion to porcine buccal tissue compared to vesicle structures [64]. The mucoadhesive hexosomes described were formed with various surfactants, including Pluronic® F-127, Pluronic® F-68 and Pluronic® F-108. Pluronic® F108 showed similar mucus-penetrating properties to Pluronic® F127 [158]; however, Pluronic® F-68 did not sufficiently coat the particle surface and was more mucoadhesive [128]. The LC cubic and hexagonal phase structure may provide mucoadhesive properties, regardless of the surface stabiliser present, suggesting the LC phase is powerful to control the interaction with mucus. Further controlled studies in (infection-associated), mucus-rich environments are required to understand the mucus interaction of LCNPs, in which adhesion may act as a reservoir to increase antibiotic concentration across the mucosal barrier.

5.4. Triggered antibiotic release from lipid particles in the presence of bacteria

Drug delivery systems can further revolutionise antibiotic therapy through either passively or actively targeting a specific area of the body where the antibiotic is required. The specific surface chemistries and size parameters of drug delivery systems facilitate passive accumulation without relying on a stimulus. These drug delivery systems have been extensively reviewed elsewhere in association with the Enhanced Permeability and Retention (EPR) effect [159, 160]. A significant shortcoming of these passive targeting systems is the lack of absolute specificity in where they may accumulate, which leads

to unwarranted side effects [160]. In comparison, active targeting drug delivery systems rely on an explicit tissue functionality or condition to either achieve particle accumulation at a highly specific site or selective release of drug from the carrier at the site of action [159]. Systems that respond to an external stimulus are termed ‘triggered drug delivery systems’ (TDDS), designed to deliver an appropriate amount of drug directly to the intended site and minimise off-target side effects [161].

Bacteria change the physiological microenvironment through the release of a range of compounds, as represented in the schematic in Figure 11. These specific environmental changes can be harnessed to design a TDDS to respond to a bacterial infection specifically. For example, *P. aeruginosa* produces biosurfactants (i.e. mono- and di-rhamnolipids) that increase virulence, motility and stimulate biofilm formation. The release of amikacin from Arikayce® is triggered by the biosurfactants solubilising the liposome [130, 162]. There are numerous secretion products of bacteria, including toxins that promote pathogenicity of the organism, and hydrolases that obtain nutrients and invade the host organism [163]. Other secretion products can change the microenvironment of the host, e.g. the local pH or temperature [164, 165]. These factors, which depend on the presence of an infection, provide multiple opportunities for targeted and triggered release of antimicrobials for a specific bacterial TDDS.

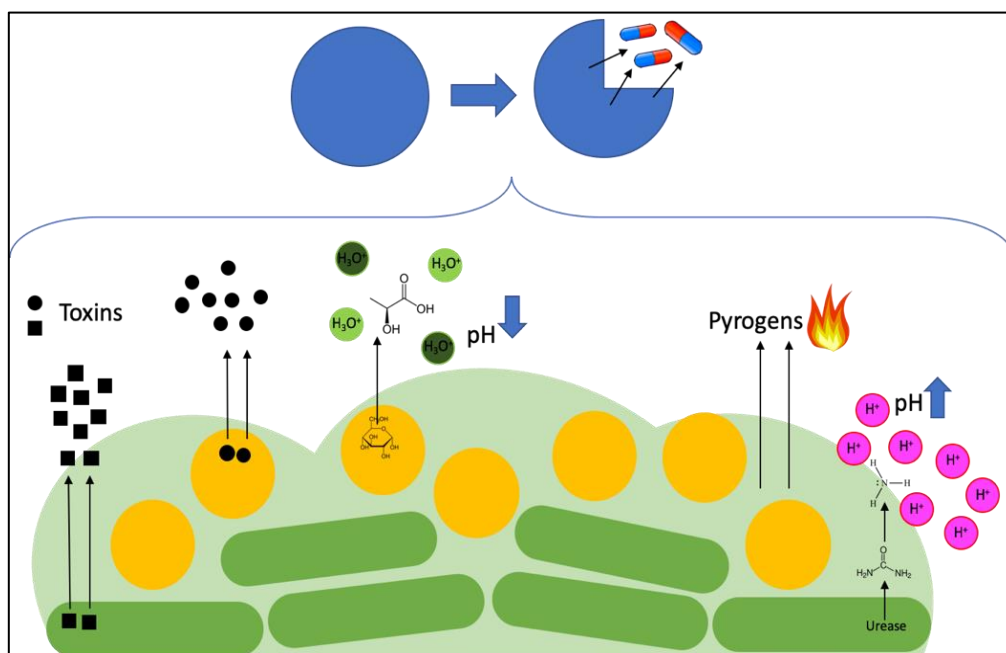


Figure 11. Bacteria represented in a biofilm with potential triggers for a drug delivery system, including toxins produced by the bacteria, alternations in the environment pH surrounding the infection, productions of pyrogens increasing the (local) body temperature

5.4.1. Digestion by bacterial toxins as a mechanism for triggered release

Bacterial toxins elicit damage to the host, exerting dominance of the bacteria and permitting survival [166]. There are an array of toxins, such as exotoxins secreted by the bacteria and endotoxins (otherwise known as lipopolysaccharides), that constitute the bacterial cell outer membrane and are secreted upon lysis of bacterial cells [167]. Exotoxins are comprised of type I cell surface-active toxins (e.g. super-antigens and neurotoxins), type II membrane damaging toxins (e.g. pore-forming toxins and lipases) [168], and type III intracellular and extracellular matrix toxins (e.g. hyaluronidase and elastase)

[163]. During the early stages of infection, toxins inform the host of the presence of bacteria by raising inflammatory markers, causing tissue damage and disabling the host immune systems [165]. If the immune system does not clear the bacteria (and toxins), the toxins cause fatal pathologies, such as toxic shock syndrome and sepsis [167].

One class of toxins are digestive enzymes that catalyse the hydrolysis of esters or amides. Many excipients utilised as drug delivery systems contain the same functional groups [169, 170]. Enzymatic digestion of the excipient by the secreted enzymes can facilitate a triggered drug release and is a prime example of a bacterial TDDS. **Table 4** details different drug delivery systems triggered by bacterial toxins, described for antibiotics.

Table 4. Different bacterial triggered lipid-based delivery systems explored.

Trigger	LBDDS	Trigger mechanism	Ref
Alpha toxin	Liposomes stabilised by chitosan gold nanoparticles	Bacterial toxin forms pores in liposomes membrane	[171]
Lipase	Lipid coated mesoporous silica nanoparticle	The shell of lipid-coated mesoporous silica nanoparticles is digested by lipase, opening the pores for antibiotic release	[172]
Lipase	LCNPs	Lipases digest monoolein-based LCNPs, degrading the LC structure	[173, 174]
Phospholipase A2 toxin	Liposomes stabilised by gold chitosan nanoparticles	Bacterial toxin forms pores in liposomes membrane	[175]
Switch from pH 7.4 to 6.5	Liposomes	The collapse of electrostatic interaction between pH-sensitive lipids in liposomes bilayer	[176, 177]
Switch from pH 7.4 to 6.5	Solid lipid nanoparticles	The collapse of electrostatic interaction between lipid in SLNs	[178]
Switch from pH 7.5 to 5.5	Liquid crystals	LC phase change between the hexagonal phase (pH 7.5) and cubic phase (pH 5.5)	[179]
Switch from pH 5 to 7	Liquid crystals	LC phase change between the hexagonal phase (pH 5) and cubic phase (pH 7.4)	[180, 181]

The lipases produced by bacteria are extracellular enzymes fundamental for nutrient supply and pathogenicity [169]. Critically important organisms of *Staphylococcus* and *Pseudomonas* produce lipases that block and disarm the host immune system, thereby enhancing the disease pathogenesis [182]. Lipases are hydrolytic enzymes that target the carboxyl ester bonds found in acyl-glycerols to liberate fatty acids and glycerol. The hydrolytic activity occurs at the lipid-water interface and is stereo-selective for the carbon position on the glycerol substrate (denoted as *sn*-1, 2, or 3), as represented in

Figure 12 [183]. Consecutively, *sn*-1/3 lipases liberate fatty acids positioned at the primary or tertiary hydroxyl position on the glycerol backbone, and *sn*-2 at the secondary hydroxyl position. Bacterial lipases are denoted by either having no specific stereo-selectivity (e.g. *S. aureus*) or hydrolysing primary ester bonds (i.e. *sn*-1 and *sn*-3 selectivity) (e.g. *P. fluorescens*) [169, 184].

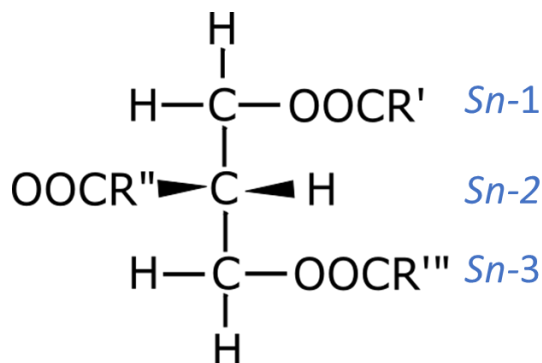


Figure 12. Fischer projection of triacylglycerol with the designated stereo-selectivity

Assembling LBDDS from glycerolipids therefore offers an opportunity for the development of bacterial lipase-triggered drug delivery systems. *Pseudomonas* lipase mediated digestion of a monoolein-based lyotropic liquid crystal (LC) gel and enhanced the release of the encapsulated gentamicin and biofilm-dispersing enzyme, alginate lyase (AL) [173]. The monoolein-based LCs were evaluated *in vitro* against *P. aeruginosa* biofilms and maintained the activity of AL with a 100-fold reduction in bacterial load [173].

Nanoparticle-based LC systems have a greater surface area, enabling an opportunity for increased rate and extent of digestion and therefore release of cargo. Monoolein-based particles were more digestible than the bulk LCs due to the substantial increase in surface area, promoting higher activity of the interfacial enzyme [174]. As lipases digested monoolein, the LC structure was degraded, triggering the release of AL and small molecular weight hydrophobic antibiotics, such as rifampicin, which are otherwise confined in the LC structure. For hydrophilic compounds such as gentamicin and ciprofloxacin, the release is already rapid from the LC nanostructure and cannot be further enhanced by a trigger mechanism [174]. Moreover, non-stereoselective enzymes resulted in complete digestion of the LC structure, unlike *sn*-1,3 stereo-selective lipases [174]. The monoolein-LCNPs were susceptible to degradation by gastric and pancreatic lipases limiting their use to the local administration at an infection site. Since lipases cannot digest phytantriol-based LCNPs, the release of antibiotics could not be triggered in the presence of bacteria. However, others have shown that the addition of triglycerides in phytantriol-based LCNPs can create alternative digestion-triggered changes in the liquid crystalline structure and could be implemented for other antimicrobial compounds [185, 186].

Phospholipids used to construct liposomes, such as DSPC, are more susceptible to lipase-mediated hydrolysis than ethylphosphocholine building blocks [187], presenting opportunities for tailored liposome-based triggered systems that are responsive to bacterial phospholipase [188]. Phospholipases specifically target phospholipids in the cell membrane, thereby compromising the integrity of host cell membranes [182]. Alpha-toxin is a phospholipase produced by *Staphylococcus aureus* that causes haemolysis and tissue damage through pore formation [189]. The toxin is a 293

protein monomer that significantly increases the virulence of *S. aureus* by insertion into the phospholipid-based cell membranes, causing apoptosis [190]. The alpha-toxin triggered system is more specific to bacterial infections than lipases-triggered systems, which have similarities to human lipases [169]. By pore formation in the liposome bilayer, alpha-toxin triggered the release of vancomycin *in vitro* as a methicillin-resistant *S. aureus* (MRSA) bacterial TDDS [171]. Liposomes prepared from DSPC and 1,2-dioctadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG) were stabilised by gold nanoparticles functionalised with chitosan to prevent fusion and vancomycin leakage. The stabilised liposomal system maintained the antibacterial activity of vancomycin against MRSA *in vitro*. Prominently, the release of vancomycin from the nanoparticles was also triggered by phospholipase A toxin produced by *Helicobacter pylori*, which digested the DSPG [175].

5.4.2. Bacterial environment effects - pH triggered release

Although less specific than the enzymatic environment at the site of infection, differences in environmental conditions such as pH can also be utilised in the development of TDDSs. The pH of blood and plasma is tightly regulated at pH 7.4, while the pH of the skin, stomach and bladder is acidic (i.e. pH 2-5.5) facilitating protection against microorganisms [191]. Consequentially, the pH surrounding an infection site, specifically with bacterial colonisation, is variable. The bacterial species, site of colonisation and the host inflammatory response all contribute to the pH surrounding a bacterial infection [167, 192]. Common pathogenic bacteria (e.g. *P. aeruginosa*, *Escherichia coli* and *S. aureus*) are more likely to colonise wounds at pH values higher than physiological (i.e. 8.5, 8 and 7.5, respectively) [193]. While once an infection is established, the bacteria may decrease the pH through by-products or via the increased immune cell activity, as observed for urinary tract and respiratory tract infections [194-196]. Bacterial biofilms further introduce another layer of variability due to pH gradients, resulting in difficulties in obtaining accurate measurements leading to ill-defined environmental pHs. In tissue-related biofilm infections, the insult to the host tissue results in inflammation and anaerobic metabolism, where an acidic environment predominates [197, 198]. Due to the variable pH surrounding an infection, that is highly site and organism-specific, there is no generalised TDDS that fits all infection sites and organism, and variable pH triggers have been explored.

pH-induced changes in the protonation state of acids or bases can result in electrostatic bond breakage, disrupting the structure of the TDDS and triggering the release of the loaded cargo. While many studies have investigated polymeric or organic-based delivery systems [199-202], LBDDS containing fatty acids or other glycerolipids presents an opportunity for triggered release at high pHs by deprotonating the acid or hydrolysing an ester group. Govender and co-workers synthesised fatty acids with secondary amines that become protonated in a slightly acidic environment (pH 6). In liposomes [176] and solid lipid nanoparticles [178], incorporation of the functionalised fatty acids resulted in a structural change at pH 6 that triggered vancomycin release. These systems furthermore decreased the minimum inhibitory concentration *in vitro* and *in vivo* in mice with an *S. aureus* skin infection [177, 178].

Multiple other LBDDS systems have demonstrated pH-triggered release in non-antimicrobial applications which could potentially be applied in this area. For example, phytantriol, sucrose stearate and oleic acid formed LC bulk phases that switched between the inverse hexagonal with limited drug

release to inverse bicontinuous cubic phase (fast release) structures upon a change in pH from 5.5 to 7.4. The pH switch triggered the release of different sized macromolecules using FITC-dextran as a model [180]. Others have made similar systems responsive to the switch between pH 2 (slow release) to pH 7 (fast release) by the addition of linoleic acid to monoolein LC systems due to the lower pK_a of the acid [181]. An oppositely triggered system (i.e. fast release at low pH) can furthermore be achieved by incorporation of a weak base, such as pyridinylmethyl linoleate, providing a slow (inverse hexagonal phase) to fast (inverse bicontinuous cubic phase) transition from pH 7.4 to pH < 5.5 [179]. Tailoring the chemical composition of LC systems to respond to the infective environment presents opportunities to produce smart systems and requires further exploration in the field of antimicrobial therapy.

6. LBDDS comparisons and clinical translations

6.1. Head-to-head comparison between LBDDS

There has been little head to head studies with different LBDDS in the antimicrobial field. One study has compared LNCs and LCNPs loaded with the antimicrobial peptide DPK-060 in a poloxamer gel [103]. The LNCs were either composed of lecithin or monolaurin with medium-chain triglycerides and macrogol (15)-hydroxystearate, as mentioned before [89] while the LCNPs were formed with monoolein, as previously described by Boge *et al.* [203]. In a murine superficial incision wound infected with *S. aureus*, the antimicrobial peptide alone in the poloxamer gel performed better than the LNCs and LCNPs [103]. Both lipid nanoparticles were applied to the wound bed in a poloxamer gel for ease of administration. The limited release (30%) of DPK-060 from the LCNPs in poloxamer gel in 24 h could explain the limited activity; however, all DPK-060 was released from the LNCs in the poloxamer gel. The *in vitro* antimicrobial activity of DPK-060 was also reduced following the loading of both the lipid nanoparticles and in the poloxamer gel. While previous studies with the LNCs and LCNPs demonstrated activity maintenance or enhancement with the antimicrobial peptide, it is possible the poloxamer gel limited the release and activity of the peptide. Longer incubation or more frequent doses may be required to achieve the desired concentration and effect of the antimicrobial peptide from those formulations.

Liposomes and LCNPs have the potential for fusion with the cell membrane of bacteria [108, 112]. In contrast, while SLNs, NLCs and LNCs can be made of lipids that are inherently active antimicrobial compounds, they have not demonstrated the ability to fuse with the lipid bilayer of mammalian or bacterial cell membranes. SLNs, NLCs and LNCs systems are usually employed for the delivery of lipophilic drugs and used to improve the biopharmaceutical characteristics of drugs with low solubility and low permeability. Given that few antibiotics fit into this category, SLNs, NLCs, and LNCs likely will have a limited role in enhancing the delivery and efficacy of antibiotics. Conversely, liposomes and LCNPs are suitable for a broader range of antimicrobial therapies, including hydrophilic, hydrophobic, and amphiphilic drugs [79, 95, 96, 99]. While liposomes suffer from low drug loading capacities or potential drug leakage, LCNPs further enable loading of a higher proportion of each drug due to the larger internal surface area of the lipid structure and great potential to fuse with lipid bilayer membranes. Liposomes and LCNPs have also demonstrated the ability to incorporate lipids into the structure with inherent antimicrobial properties. However, given the lack of vigorous preclinical and clinical studies, it

is premature to assign particular importance to one class of lipid nanoparticle to tackle infectious diseases.

6.2. Challenges and limitations in available models

Recently, the Food and Drug Administration (FDA) approved the liposomal amikacin formulation, Arikayce[®], for *Mycobacterium avium* complex (MAC) lung infections. Arikayce[®] improved the penetration of amikacin in biofilms, enhanced the activity against intracellular infections of *M. avium* [204] and overall improved the odds of patients providing uninfected sputum cultures in the Phase III clinical trial (CONVERT) [111]. The liposomal formulation of amikacin is composed of DPPC and cholesterol. Using proprietary technology, the amikacin load in Arikayce[®] (up to 40% w/w) also exceeded typical drug loads in liposomes (max 20%) by 2-fold [111, 205].

While considering the breadth of studies performed on lipid nanoparticles, besides Arikayce[®], there are no other LBDDS that have translated into the antimicrobial market. The bottleneck arises from a multitude of factors, including; discontinuation of research programs due to limitations in funding, limited correlations between *in vitro* experimentations and (pre)clinical studies, and limitations in well-planned studies. As demonstrated by the comparison between LNCs and LCNPs [103], the lipid nanoparticles in a topical gel provided no additional benefit *in vivo* compared to the gel containing the antimicrobial alone. In-depth analysis of the underlying molecular mechanisms that promote or prevent the antimicrobial activity is critical to developing the field further. In the head to head study with LNCs and LCNPs, there are many unanswered questions to why the LNCs and LCNPs did not perform. How well did the animal model correlate to human infection? Did longer or more frequent doses need to be administered? Did the gel hinder the lipid nanoparticles? Interdisciplinary and collaborative initiatives involving experts in drug delivery, materials sciences and clinical microbiology might be able to address these fundamental question and drive innovation collectively.

Moreover, adequate evaluation of the system in an appropriate and biologically relevant system is critical for future development. Without broader *in vitro* and preclinical understanding, and careful design of clinical trials, translation into clinical efficaciousness may suffer, as exemplified by Apulmiq[®] (also known as Lipoquin[®] and previously known as Pulmaquin[®]). Apulmiq[®] (Aradigm Corporation), is a liposomal formulation of ciprofloxacin [206]. In a Phase II clinical trial (ORBIT-2), the dual release of ciprofloxacin from the inhaled Apulmiq[®] resulted in a 4.2-log reduction in *P. aeruginosa* and a 76-day delay to first pulmonary exacerbation compared to placebo in non-CF bronchiectasis patients [207]. However, the delay in pulmonary exacerbation in non-CF bronchiectasis patients was non-significantly different compared to placebo in a Phase III clinical trial (ORBIT-3 and -4) [208]. Due to the discrepancies between Phase III Clinical Trials, the FDA and the European Medicine Agency (EMA) have presently declined the market approval application for Apulmiq[®] for non-CF bronchiectasis.

The paucity of well characterised, biologically relevant infectious disease models is a limiting factor for clinical development. No animal model completely replicates the human response to infection, and often a combination of models is required to understand the full pathogenesis or treatment response following infection. Mouse models are routinely employed; however, due to physiological and genetic differences

in mice infectious disease models, they can significantly differ to the infection in humans [209]. For example, a CF murine model exists with the CF transmembrane conductance regulator gene mutation, but different clinical syndromes manifest compared to humans, particularly in the pancreas and lungs [210]. There is a growing interest in using organ-on-a-chip and 3D co-cultures of bacteria and humans' cells as a more biologically relevant model while decreasing the reliance on animal models [211]. However, there is a long road to travel ahead before organ-on-a-chip and cellular co-cultures dominate over animal studies that lead into human, clinical trials.

7. Overview and future perspectives

Biomimetic and antimicrobial lipids are an excellent solution and tool to improve antimicrobial therapies against infectious diseases. Liposomes are, by far the most well-known and studied lipid nanoparticle for antimicrobial therapies. Besides targeted delivery via fusing with the bacterial cell membrane, liposomes can also be tailored as a bacterial triggered release drug delivery system. However, low drug loading and drug leakage are the primary issues of liposomes, ultimately leading to decreased shelf-life stability and incorrect or sub-therapeutic dosages in patients. In comparison, SLNs, NLCs and LNCs have improved shelf-life stability and can have intrinsic antimicrobial properties. Nevertheless, similar to liposomes, these lipid nanoparticles also have a limited ability to load large amounts of hydrophilic compounds, which includes most antibiotics. Alternative methods have been developed, such as active loading of hydrophilic compounds onto surfactant capsules or utilising lipophilic pro-drugs, and hydrophobic ion-pairing, which may be viable for future developments of SLNs, NLCs and LNCs as antimicrobial drug delivery systems [77, 78]. Yet, there is little evidence to suggest that SLNs, NLCs and LNCs are a better approach than liposomes.

The modulating effects of lipids and LBDDS on antibiotics are promising for the development of advanced, novel antimicrobial therapies. By producing enhanced synergistic antimicrobial effects with the loaded antibiotics, innately antimicrobial active LBDDSs are advantageous when high concentrations of actives are required (i.e. biofilm infections). By simultaneously employing two or more antibacterial principles against pathogens, the development of antimicrobial-resistance may also be reduced. The perfect lipid that produces a synergistic effect with antibiotics has yet to be confirmed; however, monolaurin is a prominent front runner undergoing pre-clinical and clinical trials. From the current literature, it furthermore appears that LBDDS with a submicron particle size with near-neutral surface charge, and comprised of lipids with low crystallinity or medium-chain fatty acids/monoglycerides are suitable for antimicrobial therapy to target the most notorious biofilm bacterial infection, fuse with bacteria and elicit an antimicrobial effect. While liposomes have had commercial and clinical success, development of the next generation systems could improve the range of effective antimicrobial therapies.

In this context, LCNPs have emerged as a potentially promising type of LBDDS for antimicrobials [117]. However, LCNPs have yet to be further validated mechanistically and clinically before becoming a primary strategy to improve the efficacy of antimicrobials. The unique nanostructure and functions of LCNPs can ideally combat the chemical and physical barriers of bacteria. While the fusion of LCNP has

been modelled with lipid bilayers and mammalian cells [119], direct quantification of the fusion mechanics with bacteria is required. The fusion dynamics of LCNPs may also lead to increased biofilm penetration, as demonstrated with fusogenic liposomes [212]. The limitation in understanding this phenomenon involves the complexity and skill of directly quantifying the interaction between a single bacterial cell and the complex structure of LCNPs. The responsiveness of LCNP to bacterial stimuli and the ability to incorporate antimicrobial monoglycerides might lead to the development of an “all-rounder” LBDDS. So far, these LBDDS are underexplored in the field of antimicrobials, but with advanced laboratory, preclinical and clinical investigations, they could further innovate antimicrobial therapies. The “all-rounder” LBDDS can therefore ultimately lead to an advanced antimicrobial therapy, that targets, interacts and synergistically increases the effects of loaded antibiotics, overcoming the multiple biological, chemical and physical barriers associated with bacterial infections.

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