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Functional Materials to Overcome Bacterial Barriers and Models to Advance Their Development

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With the emerging problem of antimicrobial resistance, the world is facing a slow but dangerous pandemic. While the discovery of novel antibiotics is reaching a nearly exhaustive end, new concepts for anti-infective drugs are emerging. So-called pathoblockers aim to de-weaponize bacteria rather than just killing them. As the target of these molecules is typically located intracellularly, however, hitherto almost unnoticed biological barriers are emerging such as the biofilm matrix, the bacterial cell envelope, efflux pumps, and eventual bacterial metabolism. This leads to a new paradigm that is to maximize bacterial bioavailability. To overcome the bacterial barriers, especially when further optimization of the active molecules is not possible, functional materials are needed to engineer innovative delivery systems. Those may not only enable novel anti-infective molecules to reach their targets, but will also improve the bacterial bioavailability of existing anti-infectives. Additionally, there is a need for better infection models that allow studying drug effects on both the bacteria and the host in a relevant manner as needed for rational anti-infective drug development.

1. Introduction

1.1. The Threat of Bacterial Resistance and the Need for Novel Anti-Infectives

Antimicrobial resistance (AMR) is the ability of a microbe to learn how to avoid being killed by antimicrobials. Reports of this global health threat are increasing, and it is estimated that 700000 deaths per year worldwide are attributed to AMR. The number of fatalities is predicted to increase to 10 million per year by 2050, making AMR the leading cause of death if no action is taken. The World Health Organization has categorized the different critical pathogen groups into priority lists. Notably, priority list 1 comprises exclusively multiple drug-resistant (MDR) Gram-negative bacteria (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacteriaceae*). This emphasizes the difficulty of combating these bacteria with conventional antimicrobials, mainly due to the complex Gram-negative bacterial cell envelope. The usual workflow in clinical settings starts with using some first-line agent(s), followed by second and third lines, reaching last resort alternatives that often have serious effects on the life quality of the patient. Nevertheless, time is always not in favor of the patients as confirming the resistance and initiating the new treatment protocol can happen in a very late stage after the infection has built its fortresses and castles (for instance in the form of biofilms), rendering it both resistant and tolerant to antibiotic treatment.[1] In this perspective, we will have a special emphasis on the Gram-negative cell envelope and bacterial biofilms as biological barriers. As regards the human body, we shall pay some special attention to the lungs, which are one of the organs most seriously affected by infectious diseases.

AMR is a phenomenon that occurs naturally but is propagated by extensive and inappropriate use of antibiotics. Moreover, the increasing emergence of multidrug-resistant bacteria has made the search for new antibiotics even more critical. The lack of new classes of antibiotics to fight against these resistant bacteria is a major concern. The pipeline for new antibiotics has slowed down in recent years, and the discovery void for new classes of antibiotics is becoming more and more apparent. It is noteworthy, that none of the potential antibiotics in current clinical trials represents a novel class against Gram-negative bacteria. On the other hand, there are many reasons for the lack of research on novel antibiotics, which include a lack of satisfactory return on investment, insufficient cooperation between academia, industry, and still limited public awareness and funding. However, there have been some initiatives recently, such as the "Global Antibiotic Research and Development Partnership" (GARDP).[2] To combat the problem of AMR, there is an urgent need for more

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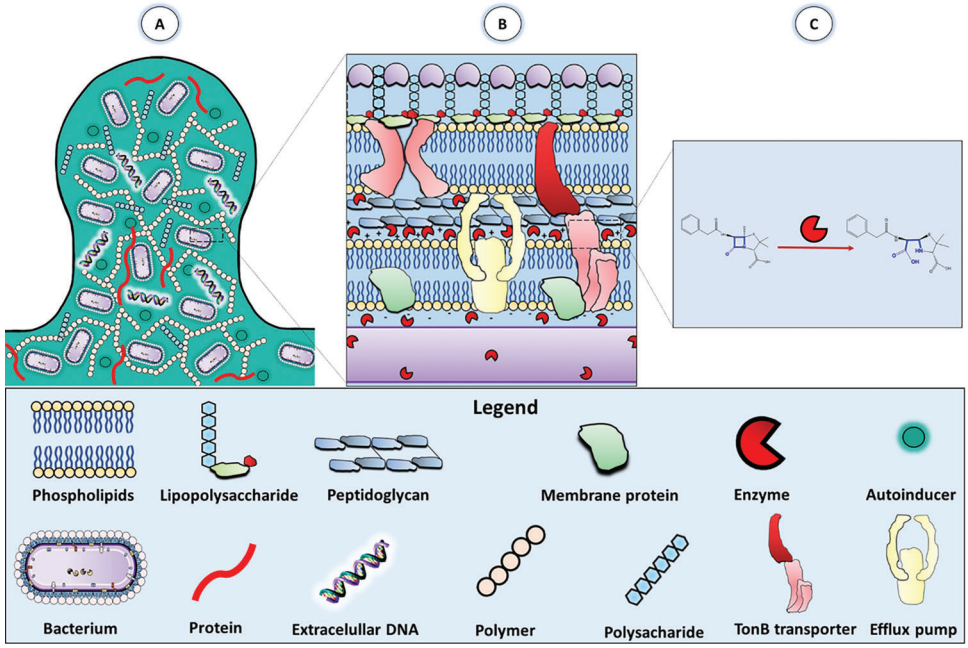

Figure 1. Biological barriers that affect bacterial bioavailability. A) The biofilm matrix, composed of extracellular polymeric substances (EPS), acting as a diffusion barrier; B) the (especially Gram-negative) bacterial cell envelope as a complex barrier comprising passive as well as active influx and efflux mechanisms; C) bacterial metabolism, caused by enzymes degrading different anti-infectives intracellularly, at the cell membrane or after secretion in the extra-bacterial environment (e.g., inactivation of Penicillin G by β-lactamases).

research not only in the field of drug discovery but also of drug delivery. Besides new functional materials, this will also require innovative test systems to verify the mode of action and potency of promising drug molecules as well as the ability of new strategies to deliver these active molecules to their bacterial targets. Complex in vitro models (CIVMs), especially when they are human-based, may be more predictive for the clinical settings than some questionable animal models and comply with the desire of the community to reduce and refine animal experiments.[3]

1.2. The Concept of Bacterial Bioavailability

When administered to the human body, drugs get initially absorbed, distributed, metabolized, and lastly excreted (ADME).[4] The rate and extent at that an active molecule reaches its biological site of action is defined as bioavailability.[5] Analogously, we have recently proposed the term "bacterial bioavailability" for referring to the amount of anti-infective that reaches the site of action inside the bacterium.[6]

Some authors have used the term "accumulation" to describe the quantity of a drug inside a bacterium.[7] It is widely accepted that factors that contribute to drug accumulation are permeation and efflux.[8,9] Nevertheless, other factors such as intracellular distribution and metabolic modifications (enzymatic degradation) of drugs might play an important role in the overall resistance mechanism.[10] In the human body, active molecules get distributed into different organs. Likewise, in the bacterium, molecules might get distributed and accumulate in the membrane, periplasm, and cytoplasm.[11] Additionally, metabolic enzymes such as, e.g., β-lactamases are unequally distributed between those subcellular compartments.[12] While such metabolic barriers have been proven essential in the displayed AMR, the specific role of bacterial enzymes and their precise localization for the development of resistance is not yet well investigated. Hence, the term bacterial bioavailability is preferred to describe the results of bacterial pharmacokinetics and underlying mechanisms that may delimit antibacterial activity and at the same time may cause antibiotic resistance.

2. Bacterial Barriers and Strategies to Overcome Them

Bacteria have evolved in a manner that controls the inlet of different substances. This is crucial to investigate because drugs need to be delivered to their targets, especially when those are located intracellularly. As shown in **Figure 1**, These bacterial barriers (biofilm, bacterial cell envelope, and metabolism) are challenging and complex, comprising both active and passive processes. The strategies to overcome these barriers vary between destroying, inhibiting the formation, or penetrating the barrier. The understanding of these bacterial barriers, their dynamics to develop resistance against anti-infectives, and how to overcome them is an important milestone for developing novel anti-infectives as well as functional materials for improving their delivery.

2.1. Biofilms

Bacteria can either live as free-floating planktonic cells or aggregate to form more complex enclosed structures known as

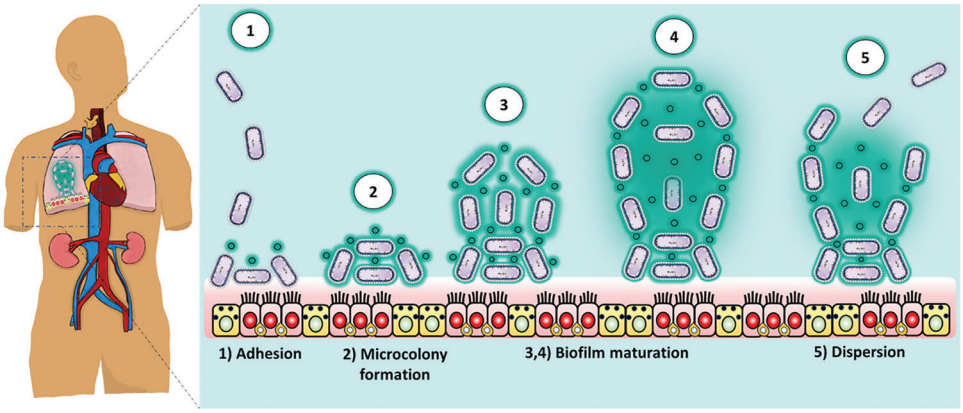

Figure 2. Stages of biofilm growth cycle in the human host (e.g. biofilm in lungs). It starts with the adhesion of bacteria to tissues (1), followed by aggregation and formation of small microcolonies (2), then the biofilm starts maturing (3) and grows into larger structures (4). Finally, the biofilm disperses partially to spread to other regions or organs (5).

biofilms.[13] The term biofilm refers to a habitat of bacteria enclosed in a matrix of self-produced extracellular polymeric substances (EPS), which makes up for over 90% of the biofilm dry mass and consists of exopolysaccharides, nucleic acids, proteins, lipids, and other biomolecules.[14] A mature biofilm represents a multifunctional biological barrier, which protects the bacteria from their surrounding environment, enhances nutrition availability, and facilitates host immune system evasion and tissue colonization. The process of biofilm formation can be divided into different stages: initial attachment of the bacteria, formation of a microcolony, maturation, and dispersion (**Figure 2**).[15] It starts with planktonic bacteria and is completed when bacterial cells escape the biofilm to return to the planktonic growth mode and initiate a new growth cycle.[16]

The whole process is coordinated by communication systems summarized under the umbrella term "quorum sensing" (QS). QS is based on signaling molecules called autoinducers (AIs) that are produced by bacteria. When the concentration of AIs produced exceeds a specific threshold, certain genes get activated resulting in the production of EPS as well as virulence factors, bioluminescence, etc. Gram-negative bacteria employ acyl-homoserine lactones (AHLs) as signaling entities, referred to as autoinducer-1 (AI-1). The QS system of Gram-positive bacteria on the other hand employs auto-inducing peptides (AIPs). Apart from AI-1 and AIPs, a third signaling molecule represented by AI-2, can be produced by both, Gram-positive and Gram-negative bacteria.[17]

Nowadays, it is estimated that 80% of the chronic bacterial infections in humans are biofilm-related, and are well-recognized in the medical practice ranging from medical devices-related infections to superficial (e.g., wounds) and inner tissue infections.[18] Because biofilms act as a physiochemical barrier toward anti-infectives, they make some substantial contribution to the occurring antibiotic resistance. As the crucial role of biofilm is recognized, efforts are being made to understand the biochemical implications and to develop viable strategies for overcoming this biological barrier. Such strategies include the destruction of the biofilm matrix, inhibition of the biofilm formation, and penetration of the biofilm as a tool for delivering destructive or inhibitory anti-biofilm agents (**Figure 3**).

2.1.1. Destruction of Mature Biofilms

The destruction of mature biofilms refers to the disintegration of the EPS matrix with or without killing the embedded bacteria. Biofilms can be disrupted by thermal or physical interventions which, however, may not be easily translated to the clinic. Another intervention is the production of effervescent gases inside or near the biofilm, like H_2O_2 or NO. Nanoparticles releasing these gases upon contact with the biofilm may be promising in this context.[19] Chemical disruption is another approach in which the surfactant properties of the chemicals can help the antimicrobials penetrate the biofilm matrix to reach the bacteria.[20] Antimicrobial photodynamic therapy has also drawn the attention of researchers. However, the practical application of such a therapy, especially for infections in inner organs may be limited. The antibiofilm strategies along with the advantages and limitations of novel drug molecules have been comprehensively reviewed elsewhere.[21]

The Biofilm Matrix as Target: During the dispersion phase of the biofilm, an increase of secreted extracellular enzymes such as glycosidases, proteases, and DNases is observed.[22] The secreted enzymes demolish parts of the EPS matrix to release some of the embedded bacteria to colonize new sites. As illustrated in Figure 3, scientists have been trying to investigate their capacity in destroying mature biofilms, without killing the bacteria, but rather by making them accessible for treatment. For instance, recombinant Dispersin B, a glycosyl hydrolase found in *Aggregatibacter actinomycetemcomitans* can disrupt poly-N-acetylglucosamine and shows antibiofilm activity against *S. epidermidis* biofilms, where this is the main exopolysaccharide of the biofilm matrix.[23] Other enzymes with the ability to degrade biofilm matrix are alginate lyase and deoxyribonuclease I (DNAse I). When vancomycin was combined with DNAse I to treat *Enterococcus faecalis* biofilms, which are rich in eDNA, the minimum biofilm eradication concentration (MBEC) was at the same level as for planktonic bacteria, thus indicating the cooperative anti-biofilm activity of this enzyme.[24] The latter was approved for clinical use in 1993.[25] Likewise, the addition of alginate lyases to vancomycin displayed an 8-fold reduction of the MBEC of vancomycin against the biofilms of *Enterococcus*

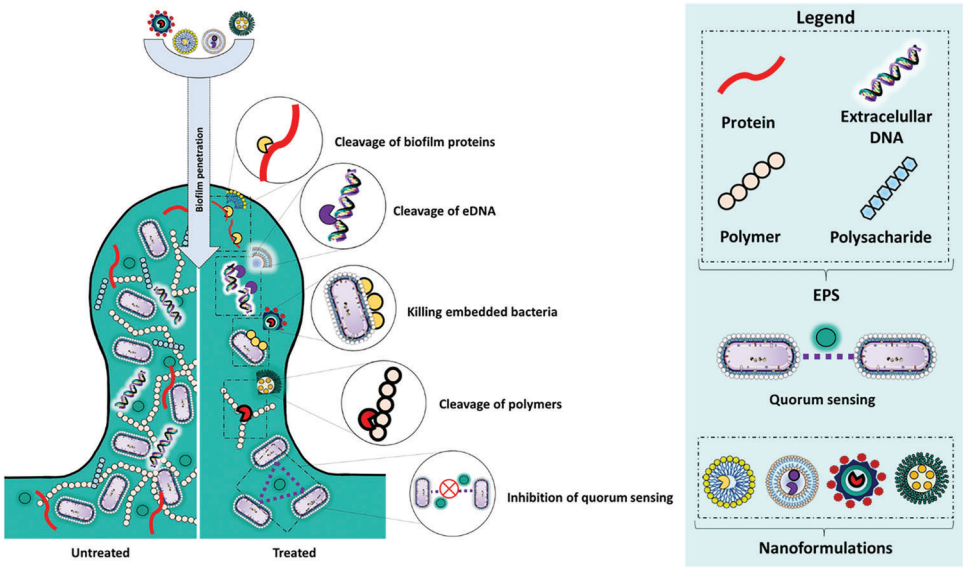

Figure 3. Strategies to overcome the biofilm barrier. The figure depicts the penetration of the biofilm by different nanocarriers loaded with anti-microbial agents. The anti-microbial agents can induce the destruction of mature biofilms by targeting the biofilm matrix and/or the embedded bacteria (e.g., cleavage of eDNA and proteins, hydrolysis of the matrix sugars, demolishment of the bacterial membrane), and inhibition of biofilm formation (e.g., by inhibition of quorum sensing).

faecium (rich in alginate). The latter was also proven to enhance the antibacterial activity of aminoglycosides against the biofilms of 13 different strains of *P. aeruginosa*.[26] Small molecules such as cis-2-decenoic acid, a medium-chain fatty acid chemical messenger produced by *P. aeruginosa* can also induce the dispersion of biofilms of *Staphylococcus aureus*.[27]

Another promising approach to destroy mature biofilm matrices is by repurposing so-called mucolytic agents. These agents are normally prescribed to enhance mucus clearance from the respiratory tract. However, due to the high similarity in the composition of mucus and some bacterial biofilms, mucolytic agents are also effective in destroying these biofilms. Some clinically approved mucolytics like ambroxol disrupt bacterial biofilms, although the exact mechanism of action is not known.[28] *N*-acetyl cysteine, which is in clinical use for facilitating the expectoration via mucolysis, has also been proposed to exhibit its anti-biofilm action by disrupting disulfide bonds and is thus active in disrupting biofilms with disulfide-rich moieties.[29] Mucolytic enzymes and other agents do not possess intrinsic antimicrobial activity, but are already clinically approved for other indications. Therefore, repurposing them in combination with some approved antibiotics should be relatively straightforward from a regulatory point of view.

Embedded Bacteria as Target: Similar to planktonic bacteria, also bacteria engulfed within the biofilm matrix can be the target of conventional antibiotics. EPS, however, acts as a physiochemical barrier that decreases the antimicrobial activity of antibiotics either by binding to them and/or by limiting the penetration through the biofilm matrix. To overcome this barrier, researchers are making efforts to discover new materials or repurpose existing ones. Such is the case of antimicrobial peptides (AMPs). AMPs are mostly small and amphiphilic peptides containing up to 100 amino acids with a net cationic charge and can be found in various organisms as a part of their innate immune system.[30] For instance. LL-37, an AMP found in the human body, has antimicrobial activity against both Gram-positive and Gram-negative bacteria and is similarly effective in destroying the biofilms of *S. aureus* as of *Escherichia coli*.[31] Bacteria can also produce AMPs called bacteriocins, which are effective in killing planktonic bacteria and exhibit antibiofilm activity. To illustrate, Bacin A2, identified in *Bacillus sphaericus* TL12, could compromise the membrane integrity of *S. aureus* and methicillin-resistant *S. aureus* biofilms, resulting in disassembling of the biofilm.[32] Similarly, it was found that the novel bacteriocin AMYX6 and XJS01 could impair the membrane integrity and consequently inhibit biofilm formation in *Salmonella Enteritidis* and *S. aureus* respectively.[33,34] The antibiofilm mechanism of action of AMPs, however, is possibly ambiguous. For instance, LL-37 exhibits its antibacterial activity by disrupting the membrane of biofilm-embedded bacteria.[35] On the other side, it also has antibiofilm activity against *P. aeruginosa* possibly by interfering with its quorum-sensing system.[36,37]

Despite their potential, natural AMPs have not yet been translated into clinical settings due to their susceptibility to temperature, pH, ion concentrations, and other environmental factors, as well as toxicity to host cells.[38] To overcome such limitations and to better exploit the potential of AMPs, different drug delivery systems have been and are currently under investigation. For instance, Chitosan-LL37 nanoparticles improved the antimicrobial activity of LL-37 and enhanced its stability in the presence of acidic pH, salts, and thermal treatment.[39] Additionally, the controlled release of AMPs was proven possible by the employment of smart hydrogels as a delivery strategy.[40] Despite the limitations, the current and future clinical perspectives for AMPs can be further improved by designing (semi-)synthetic analogs as well as suitable delivery systems.

2.1.2. Inhibition of Biofilm Formation

QS coordinates communication and behaviors important in the infection process including the formation of bacterial biofilms. An emerging approach to combat antibiotic tolerance of bacterial biofilms is by interfering with the QS signaling that renders the bacteria more susceptible to antimicrobial agents (Figure 3). Such interference can be achieved by employing a class of compounds known as quorum sensing inhibitors (QSIs) and pathoblockers. QSIs disrupt bacterial communication systems, by inhibiting the synthesis or activity of AIs, interfering with downstream signaling pathways, or blocking the receptors responsible for the detection of the signaling molecules.[41,42] For instance, Str7410, identified with high AI-2 QS inhibition activity, was added to the co-culture of *P. aeruginosa* and *S. aureus*, leading to a significant reduction of the swarming motility, pyocyanin and elastase production, as well as in the biofilm formation of *P. aeruginosa*.[43] Interestingly, AI-2 is produced by both Gram-negative and Gram-positive bacteria. Therefore, any interference with the production and/or secretion of AI-2 could be a viable strategy to treat multispecies infections. Meanwhile, pathoblockers specifically block the activity of virulence factors or other pathogenic pathways. The goal is not to kill but to disarm pathogens by interfering with bacterial invasion, adhesion, nutrient acquisition, toxin production, or other virulence-associated mechanisms. To illustrate, microbial adhesion and biofilm formation in *P. aeruginosa* are usually mediated by lectins.[44] Therefore, inhibiting the adhesion process by using compounds that imitate lectins and compete with their action could be a viable pathoblocking strategy.[45]

Materials and Strategies to Overcome QSIs Limitations: Significant effort has been made toward the discovery and investigation of the effectivity of QSIs as antibiofilm agents. For instance, itaconimide 3-methylene-1- tetradecylpyrrolidine-2,5-dione interrupts the QS of *P. aeruginosa* by targeting the las system.[46] B-11, a novel QSI, could reduce the production of virulence factors such as rhamnolipids and pyocyanin under phosphate limitation.[47] Interestingly, many QSIs have emerged, but up to date, there is no QSI approved for clinical use. The reason is closely related mainly to their high hydrophobicity resulting in low solubility and therefore insufficient penetration of the biofilm matrix. To overcome this limitation several strategies have been explored including re-functionalization of QSIs, implementation of prodrug designs, combination therapies, as well as nanocarriers. In this context, silver and gold nanoparticles have been shown to display QSI activity against a range of bacterial species.[48,49] Their small size and high surface area-to-volume ratio make them effective at penetrating bacterial cells and disrupting QS signaling pathways. Also, lipid nanoparticles display promising potential as drug-delivery vehicles to combat bacterial biofilms.[50] There are also polymer-based nanoparticles that are biocompatible, biodegradable, and can be tailored by adding functional groups to modify their properties. For example, polyethylene glycol (PEG) coated nanoparticles have been shown to inhibit QS, while functionalized chitosan (CS) nanocarriers can inhibit QS and virulence in several bacterial species.[51,52] Polymeric nanocarriers can also be modified in a bioresponsive manner. For instance, alginate nanoparticles were specifically modified to deliver ciprofloxacin in combination with a QSI.[53] Other polymers, such as poly (ethyl acrylate), poly(lactic-co-glycolic acid), chitosan, and dendrimers have been found promising due to their biocompatibility, increased stability, tailored properties, controlled release, and scalability.[54,55] However, these materials need to be further optimized, and their safety profile should be further evaluated in preclinical and clinical research.[56]

As a recent example from our Institute, 3-(Aminomethyl)−2-(hexylthio)−6-nitro-4(1H)-quinolinone ("QSI (1)") is a potent pqsR inverse agonist for combating *P. aeruginosa* infections, but rather lipophilic so that it might get stuck in the biological barriers of mucus and biofilm matrix by hydrophobic interactions.[57] However, the co-encapsulation of QSI (1) and cationic tobramycin in self-assembling squalene hydrogen sulfate nanoparticles (SqNPs) could not only improve the delivery of the cationic tobramycin through the negatively charged biofilm barrier, but also helped overcome the hydrophobicity-problem of the QSI (1). SqNPs also improved the inhibition of pyocyanin compared to free QSI (1). Additionally, the drug-loaded SqNPs showed improved biofilm penetration and in combination with another, more potent QSI molecule, led to the complete eradication of *P. aeruginosa* biofilms at a concentration 64-fold lower than free tobramycin alone.[55,58]

2.1.3. Penetration of Biofilm and Mucus

The biofilm barrier is especially prominent in chronic lung infections. The latter is usually further complicated by tracheobronchial mucus. This is particularly relevant in the case of cystic fibrosis, where bacterial biofilms are embedded and can persist in the mucus, which is extremely thickened as a consequence of this genetic disorder (lack of functional chloride channels). Drug molecules thus, must penetrate both thickened mucus and EPS to reach their bacterial targets. The biofilm composition includes polysaccharides, proteins, lipids, and extracellular DNA at different ratios varying from bacterium to bacterium. For instance, the biofilm matrix of *Cutibacterium acnes* is composed of 62.6% polysaccharides, 9.6% proteins, 4.0% DNA, and 23.8% other compounds accounting for the dry weight of EPS.[59] Similarly, the mucus is composed of water, DNA, lipids, glycoproteins, and cell debris. Under normal conditions, mucus contains up to 97% water and 3% solids (mucins, non-mucin proteins, salts, lipids, and cellular debris).[60] The water component makes up more than 70% of the biofilm matrix compared to > 95% of the mucus. Both mucus and EPS are negatively charged. Therefore, positively charged anti-infectives like tobramycin are trapped on the biofilm surface, while hydrophobic antimicrobials will be repelled. Furthermore, these barriers can simultaneously alter the characteristics of the nanocarriers or other employed drug delivery systems. Thus, the penetration will depend on the physicochemical properties of the nanoparticles, the mucus, and the biofilm matrix.

Based on the ability of mucolytics to clear the mucus and disrupt the biofilm matrix, we hypothesize that the biofilm matrix exhibits similar rheological properties to mucus. If the hypothesis is correct, then the exhaustive information obtained from numerous studies on mucus rheology can be extrapolated to biofilm entities and open some new perspectives.[61,62] For

instance, PEG-coated nanoparticles previously reported to display decreased mucoadhesive behavior would theoretically also show decreased biofilm adhesion.[63] In a study, it was found that the acidic mucus milieu promotes the interaction between the surface of nanoparticles and mucins, which are the main component of the mucus layer and have been reported to attenuate the virulence of *P. aeruginosa*.[64] On one hand, surface-PEGylated solid nanoparticles exhibited the advantage of mucus penetration, while on the other side positively charged amine-modified solid nanoparticles and carboxyl-modified particles were trapped in the negatively charged mucus.[65] In another study, it was found that ultra-small (< 100 nm) solid lipid nanoparticles with hydrophilic surface properties enhance mucus penetration.[66] If the claim that mucus and biofilms display similar rheological properties stands, then it can be speculated that lipid nanoparticles and PEG-shell-modified nanocarrier could potentially facilitate biofilm penetration. Indeed, a recent study showed that PEG–PLGA nanoparticles had enhanced biofilm penetration.[67]

Materials and Strategies for Biofilm Penetration: Different nanoparticles have been shown to display biofilm penetration activity (Figure 3). Among them, polymeric nanoparticles display the greatest potential.[68] For instance, cationic polymer conjugates could penetrate through biofilm layers.[69] Likewise, biofilm-responsive caged guanidine nanoparticles could penetrate and accumulate in bacterial biofilm.[70] Other examples include CS, poly(lactic-co-glycolic acid, and polycaprolactone nanoparticles. CS is a polymer of special interest due to its intrinsic antibacterial activity.[71,72] The overall positive charge enables it to electrostatically bind to the negatively charged outer membrane of the bacteria and negatively charged biofilm EPS. Therefore, many nanoparticles were designed to be coated with CS to facilitate the biofilm diffusion of both drugs and nanoparticles.[73,74] For instance, a chitosan-polyethylene glycol-peptide conjugate with a size of ≈100 nm resulted in an increased biofilm penetration.[75] Alternatively, lipid nanoparticles could enhance the penetration of biofilms. A recent study showed that tobramycin-loaded lipid liquid crystal nanoparticles could significantly enhance the penetration and eradication of *P. aeruginosa* biofilm infections.[76]

In the context of biofilm penetration, an alternative approach might be to treat the biofilm matrix as an "ally" and employ agents that display similar characteristics to EPS polymers to coat and make loaded drugs "invisible" to the biofilm surface. The latter could be considered a biomimetic drug delivery strategy. To advance this hypothesis, several steps must be taken. First, potential materials such as polymers of the biofilm matrix should be identified and characterized. Afterward, polymeric analogs of these materials must be found or synthesized. Lastly, once available the newly identified EPS-mimetic compound can be employed as a coating agent to merge with the biofilm matrix as part of its structure and to release the cargo in the immediate vicinity of its bacterial targets.

2.2. Overcoming the Bacterial Cell Envelope

A major cause of bacterial resistance to antibiotics is attributed to the bacterial cell envelope, which represents with its complex structure a significant barrier for the internalization and accumulation of antibiotics in bacterial cells and thereby limits bacterial bioavailability (**Figure 4**). Depending on the structure of their envelope, bacteria are classified as Gram-positive and Gram-negative. The cell envelope of Gram-positive bacteria is constituted of an inner lipid membrane and a thick peptidoglycan layer representing a gel-like mesh structure. In contrast to their Gram-positive counterparts, Gram-negative bacteria have a much thinner peptidoglycan layer and confine an additional outer membrane made of an inner leaflet of lipids and an outer leaflet of lipopolysaccharide (LPS). Some Gram-negative bacteria might also have an extra outer layer called the S-layer, made of proteins or glycoproteins.[77,78] Different proteins are embedded within the bacterial cell membrane to coordinate the facilitated diffusion and transport through the membrane.

Targeting intracellular components requires the active molecule to possess the size and other physiochemical properties needed for uptake while still maintaining binding affinity and activity at the target. Besides optimizing the transport properties of anti-infective molecules, an alternative strategy is to look for materials that can serve as carriers, adjuvants, or are even antimicrobials by themselves. Such material has to interact with the bacterial membrane to either selectively target it or destroy it. It can be as little as small molecules or polymers or even much larger, such as nanoparticles, fibers, membranes or coatings, and scaffolds.[79] We will focus more on zero-dimensional materials, e.g., nanoparticles, which are favored to overcome biological barriers.

Simple Passive Diffusion: The transport of small molecules across the outer membrane of Gram-negative bacteria may occur through protein channels and/or the membrane lipids. The latter is referred to as simple passive diffusion. As with any other diffusion barrier, the main determinants are the concentration gradients and the size of the molecule in the host settings. LPS is the main limiter of simple passive diffusion, which is absent in Gram-positive bacteria. LPS consists of lipid A, inner core sugars, outer core sugars and O-antigen tightly bound together with divalent cations (Ca^{+2} and Mg^{+2}). The next layer is the peptidoglycan that is a gel-like mesh, then the lipid bilayer of the inner membrane. Only small hydrophilic neutral molecules like amino acids, water, and soluble gases are most likely to readily pass through the LPS layer.[80,81]

Facilitated Passive Diffusion: Facilitated passive diffusion is a process by which the bacteria use non-active channels to transport molecules from the outside to the inside of the bacterium without the expense of energy in the form of ATP or any potential gradient. The process depends primarily on the concentration gradient of the molecules of interest. Additionally, mutations can alter the function of these channels or porins resulting in reduced effectivity of the small organic molecules. This phenomenon has been observed in different strains, especially in *P. aeruginosa, A. baumannii*, and *Klebsiella pneumoniae*.[82]

Active Transport: Active transport refers to a process by which certain molecules are transported across the cell membrane independent of the concentration gradient. In bacteria, the so-called TonB-dependent transport system has evolved to internalize molecules that cannot translocate by facilitated diffusion, but at the expense of energy in the form of ATP or proton gradient. TonB transport is initiated when the substrate binds to one of its receptors at the OM (e.g., FhuA (binds ferrichrome),

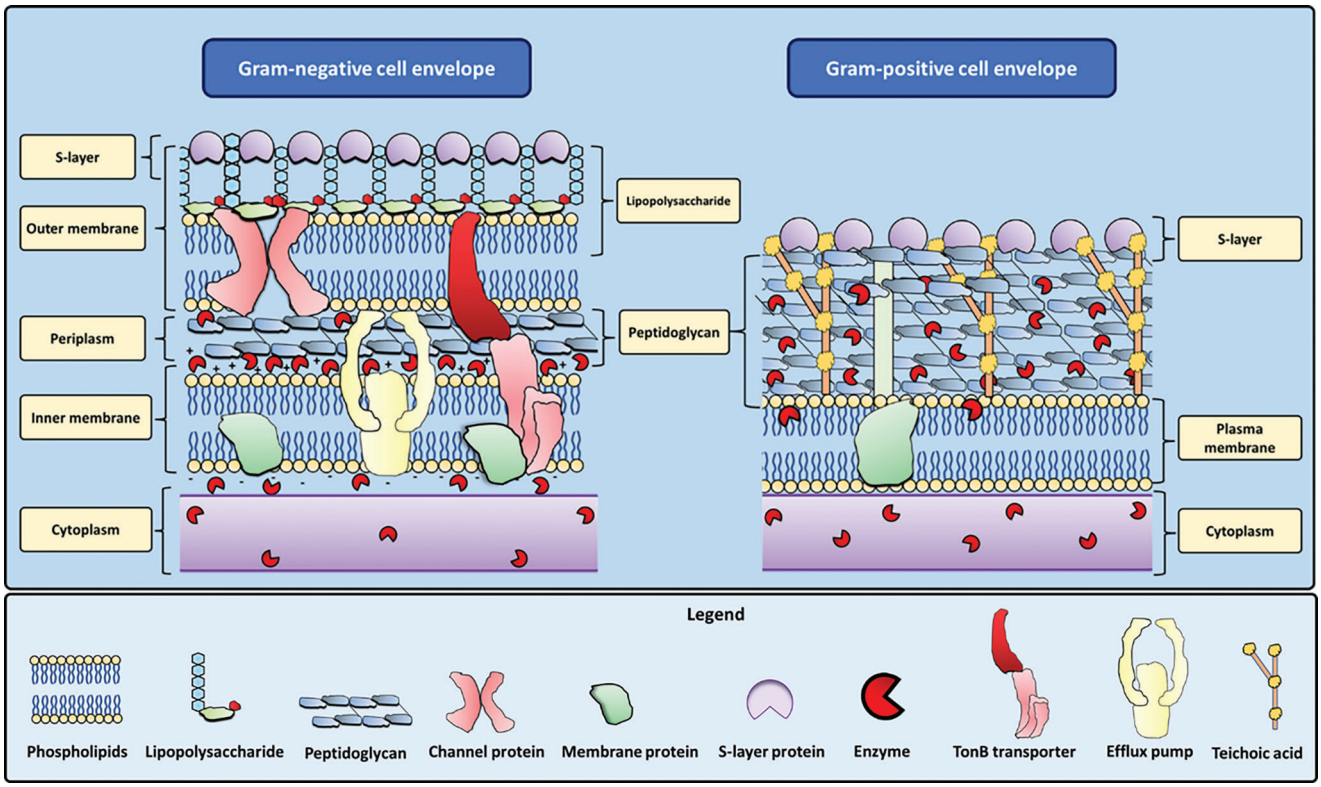

Figure 4. The different structures of Gram-positive and Gram-negative bacterial cell envelopes, illustrating the unique LPS structure as well as efflux pumps as the main limiters of simple passive diffusion. Adapted with permission.[6] Copyright 2023, Elsevier.

FepA (binds ferric enterobactin), and BtuB (binds vitamin B12)). Subsequently, the TonB system uses ATP or proton gradient for transport through the outer and inner membranes as well as the periplasm.[84]

As shown in **Table 1**, it is quite noticeable that the affinity of these channels to their substrates is variable and relatively low compared to the TonB transporter, which involves specific receptors as well as higher molecular weight cutoff. This affinity advantage of TonB-dependent transporters makes it more promising for the Trojan Horse approach for better intra-bacterium drug delivery.

Efflux Pumps: Efflux pumps are energy-dependent membrane-bound transporters that actively pump out toxic substances, drugs, and other antimicrobial agents. Notoriously they play a major role in bacterial resistance.[84] This is especially true for hydrophobic compounds (clogD7.4 > 3). On the other side, highly charged compounds with low molecular weight (<400 Da) are not affected by efflux pumps. The latter stands

Table 1. Different passive channels and TonB-dependent transporter (active transporter) in bacteria showing the substrates, the affinity (K_D) of the channel to its substrates, and an estimated size cut-off for molecules that can pass through.

Name	Substrate	K_D for a known substrate	Size cutoff [daltons]	References
Porins	Unspecific: hydrophilic molecules and ions	No/little affinity	600	[77,83–85]
LamB	Maltose, maltodextrins	10 μM for maltose	850	
BglH	Aryl-ß-D-glucoside	1–3 mM for 2-hydroxymethylphenyl-ß-glucoside	Expected as LamB due to homology	
Tsx	Nucleotides	Not determined	850	
FadL	Long-chain fatty acids	0.2 μM for oleate	300	
CymA	Cyclodextrin	28 μM for cyclodextrin	980	
TonB-dependent Transporters	Siderophores	300 nM for ferricrocin	1360	

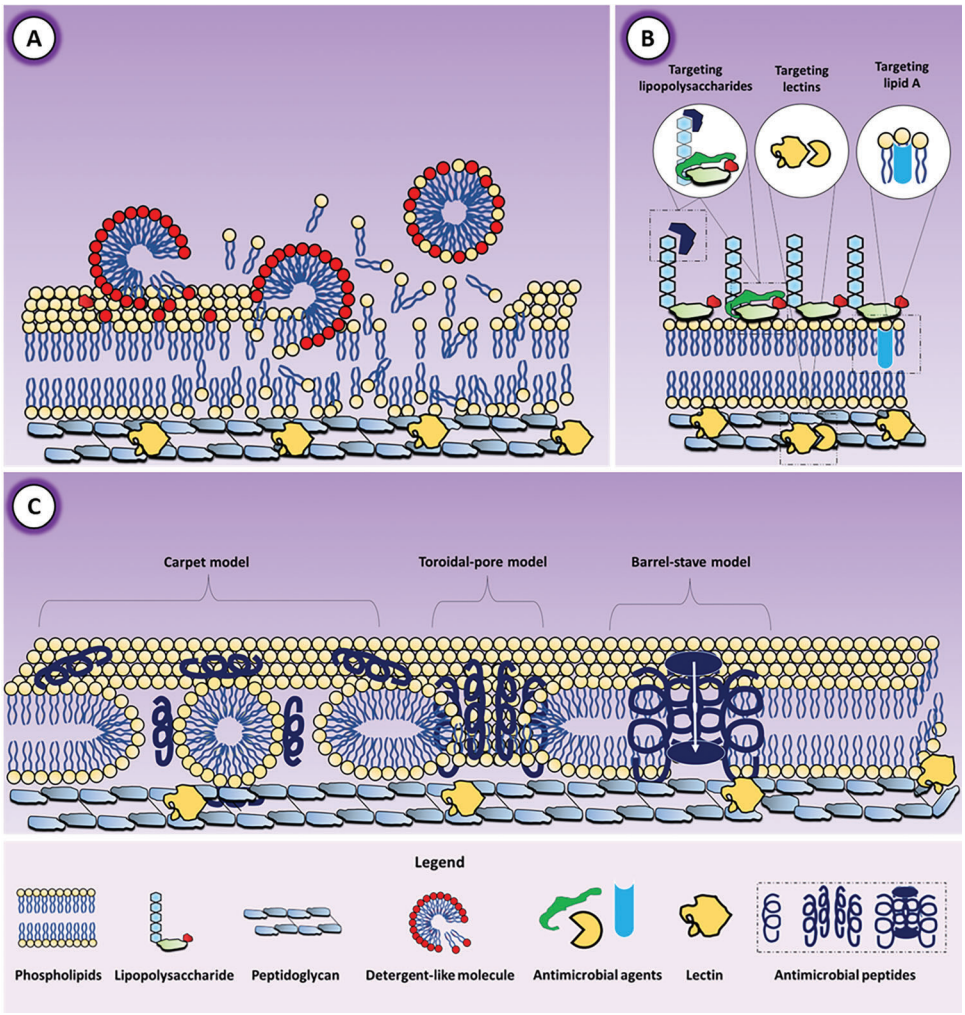

Figure 5. Strategies to enhance the permeability of anti-microbial agents across the Gram-negative bacterial cell envelope by A) Demolishment of the bacterial cell envelope (e.g., by detergent-like molecules). B) Specific bioadhesion to certain components (e.g., binding to lectins, lipid A, LPS) within the bacterial cell envelope. C) Permeabilization of the bacterial cell envelope (e.g., by membrane-active AMPs).

true also for polar zwitterions with a high molecular weight (400–600 Da). However, variations in the efflux systems among different bacterial strains like *E. coli* and *P. aeruginosa* exist, therefore, these generalizations are not absolute. Further understanding of the molecular descriptors that can be utilized to avoid efflux is important for improving bacterial bioavailability.[86] Alternatively, researchers could enhance the MIC of tetracycline by 4-fold when it was combined with efflux pump inhibitors.[87]

2.2.1. Enhancing the Permeability of the Bacterial Cell Envelope

Permeability through the bacterial cell envelope is the main delimiter of the activity of different potential anti-infectives. The permeation of small organic molecules through the bacterial cell envelope has been extensively covered in literature.[88,89] In this subsection, membrane-active materials will be discussed. We differentiate between two concepts; demolishment, and permeabilization. Demolishment refers to destroying the cell envelope to an extent that causes leakage of the intracellular components (**Figure 5**A). This is common for most disinfectants and leads to blunt killing of the bacteria. This again, however, just increases the chances of developing AMR. On the other hand, permeabilization refers to only inducing some structural changes (e.g., pore formation), rather than destroying the cell envelope. The aim is not to kill the bacterium but to facilitate the uptake of anti-infectives (e.g., modern pathoblockers) in its vicinity (Figure 5C).[90]

Demolishment: Most antibiotics that are active against Gram-negative bacteria act on intracellular targets, which requires the internalization of these molecules as a prerequisite for their activity. On the contrary, molecules that act on the bacterial cell envelope, do not require to be taken up by the bacterium to become active. Understanding this could encourage the scientific community to give more attention to membrane-directed strategies. Approaches that can be employed to demolish cellular membranes include:

- Detergents that have a hydrophobic part that sticks with the lipids and a hydrophilic part that stays in contact with the water outside the bacterium.[91]
- Peptides that can acquire α-helical conformation inside the membrane, permeabilizing the membrane.[92]
- Metal ions that can form complexes with the membrane of the bacteria, disrupting the membrane.[90]
- Gas generation within the membrane like hydrogen sulfide and H_2O_2 (which also can produce ROS to destroy the membrane).[93]

These membrane-destructive strategies need, however, to be adopted to avoid damage to the mammalian cell membrane, i.e., to target structures that are only found in bacteria, but not in mammalian cells. Such approaches include divalent cations, bacterial membrane-specific lipids and polysaccharides, and membrane overall charge. For instance, polymyxins (in use since 1964) belong to a class of antibiotics that interact with the LPS and compromise the membrane integrity of the bacteria. Nowadays, it is used as a last resort in the clinical setting because of its toxicity.[91]

So far, some detergent-like and α-helical peptides have reached advanced clinical development or are on the market. For instance, teixobactin, in addition to being a peptide-like molecule and not an AMP, targets lipid II in the bacterial membrane, inhibits peptidoglycan synthesis, and compromises the membrane integrity.[94] Other membrane-acting peptides with the ability to destroy the membrane have been isolated, e.g., melittin and maginin (reached clinical phase 3 and rejected). Melittin exhibits a detergent-like mechanism, while maginin forms an α-helix inside the lipid membrane of the bacteria. The usage of AMPs, however, is still quite limited due to the cost of production and the relatively high toxicity due to interactions with mammalian cell membranes.[95]

Nevertheless, membrane-active AMPs have enormous potential. Peptides have the highest degree of freedom and thus offer much potential to adjust the molecule toward bacterial, but not mammalian toxicity. Also, AMPs are not split structurally into a targeting moiety and an activity moiety, which therefore maximizes the antimicrobial activity normalized by molecular weight. Developing AMPs for clinical applications requires first screening against both mammalian cells and bacteria, ideally also leading to a better understanding of what governs this selectivity that has not been done until very recently.[96,97] Peptide modifications such as using D-amino acids and cyclization must also be taken into consideration. They can significantly influence the activity and selectivity of bacterial cells. In addition to peptides, synthetic oligomers, and polymers can also serve the same purpose, but more research is required to optimize their pharmacokinetics and pharmacodynamics.

Permeabilization: Permeabilization refers to the introduction of pores in the bacterial cell envelope by the employment of different pore-inducing agents. This process would therefore lead to enhanced drug uptake. Initial research could demonstrate that in some strains of Gram-negative bacteria, which had high pore expression, the minimum inhibitory concentration (MIC) of some antibiotics was decreased up to 128fold.[98] Increasing the permeability of the bacterial cell envelope would also open the perspective to convert narrow-spectrum antibiotics, acting on Gram-positive bacteria only, to broad-spectrum Gram-negative acting antibiotics. More importantly, it may enable to design agents against intracellular targets that are above 600 Daltons as long as the created pores allow their uptake.

Well-known pore-forming molecules are proteins like α-hemolysin and peptides like melittin (bee venom). Nevertheless, the proteins can cause collateral damage to mammalian cells, which limits their translation to the clinic.[99,100] However, recent studies discovered more biocompatible membrane-acting potentiators, such as polycations, cell-penetrating peptides, artificially-quaternized polymers, and combinations of the two approaches like peptide-functionalized polycationic polymers.[101–103] Others developed a relatively simple structure with two positively charged groups that interact with the bacterial membrane and were able to potentiate pre-existing antibiotics by up to 512-folds against MDR *E. coli*.[104]

Silver nanoparticles have been used for so long as antimicrobial materials because of their many involved mechanisms of action such as the inactivation of proteins (disrupting bacterial metabolism), production of reactive oxygen species, and contact interactions with the bacterial cell envelope. However, safety concerns and difficulty in excreting metal-based nanoparticles halted their further development.[105] Nevertheless, the door stays open for employing silver nanoparticles in lower doses as an adjuvant to other anti-infectives such as carbenicillin.[106]

There seem to be three crucial factors that contribute to such adjuvanted effects. First, the more cationic a molecule, the more likely it is to have antimicrobial activity. Second, the higher the alpha-helical structure of a peptide, the more rigid it will be to thus disrupt the membrane and enhance the permeation effect.[107] Third, the collective effect of more than one acting unit on the membrane can increase the activity in a near-exponential manner up to a certain limit.[108] It is worth noting that the first two factors are correlated with increased mammalian toxicity that makes it tricky to translate such materials to the clinic. In our perspective, multivalent membrane-active drug conjugates can have a much higher collective potency as all units get to exert their effect simultaneously in analogy to membrane attack complexes (MAC) embracing the biomimetic approaches.[109]

2.2.2. Binding to the Bacterial Cell Envelope (Bio-Adhesion)

Finding molecules that specifically bind to bacteria but not to mammalian cells is usually the bottleneck in developing targeted anti-infective therapies. As shown in Figure 5B, some features of the bacterial cell envelope have already been identified as targets, which may allow the development of "magic bullets" especially for Gram-negative bacteria.[110] Potential targets for achieving bio-adhesion to bacteria are:

- The overall pronounced anionic character
- LPS molecules, which form the outer leaflet of the outer membrane
- Divalent cations that are needed to stabilize the LPS
- Lipid A, as a specific component of LPS which is not found in mammalian cells
- The lectins; LecA and LecB

In addition to more or less specific binding, some molecules can also destroy the bacterial, but not the mammalian cell

Table 2. Examples of innovative materials and applications involving specific binding (bio-adhesion) to Gram-negative bacteria.

Target	Localization	Material	Regulatory status	Mechanism	Application	Reference
Lipid A	Inner leaflet of the LPS	Lipid A analogs, e.g., eritoran	Failed in phase 3	Mimics Lipid A to disrupt the outer membrane	Gold clusters	[113,114]
LPS	Outer membrane	Polymyxins, e.g., colistin and polymyxin B	Approved	Binds to and compromises the LPS	PMB-loaded cubosomes	[115,116]
LecA and LecB	Periplasmic space	Carbohydrates	Investigational	Blocks LecA and LecB	Surface modified polymeric nanoparticles	[117,118]
Peptide-membrane interactions	In between the lipid molecules	Membrane-active peptides, e.g., PEGylated LL-37	Clinical phase 2	Interact with the bacterial membrane	Photodynamic NP decorated with TAT	[103,119,120]

membranes. The selective destruction of membranes takes place by crystallization,[111] hydrophobic interactions with membrane lipids, formation of ion-permeable channels in the bacterial membrane by peptide-assemblies, and peroxidase-like activity via the release of H_2O_2 into the membrane.[101,112]

As **Table 2** illustrates, such specific binding materials are often employed to decorate the surface of nanocarriers to make them bio-adhesive to bacteria and to release their cargo in close vicinity to their target. Researchers can design a carrier compatible with the cargo and the needed loading capacity, as shown in **Figure 6**. A wide variety of nanoparticles have been created with different combinations and increased complexity, for example by loading a second active molecule in the coating or by using the polymeric core as an active antimicrobial.

Among pharmaceutical polymers, CS is widely used both as a carrier and because of its bioadhesive and antimicrobial properties. Due to its pKa 6.5, it has the advantage to acquire a positive charge in slightly acidic pH that is likely to happen in the inflammation sites (pH 5–6). The combination of CS with other

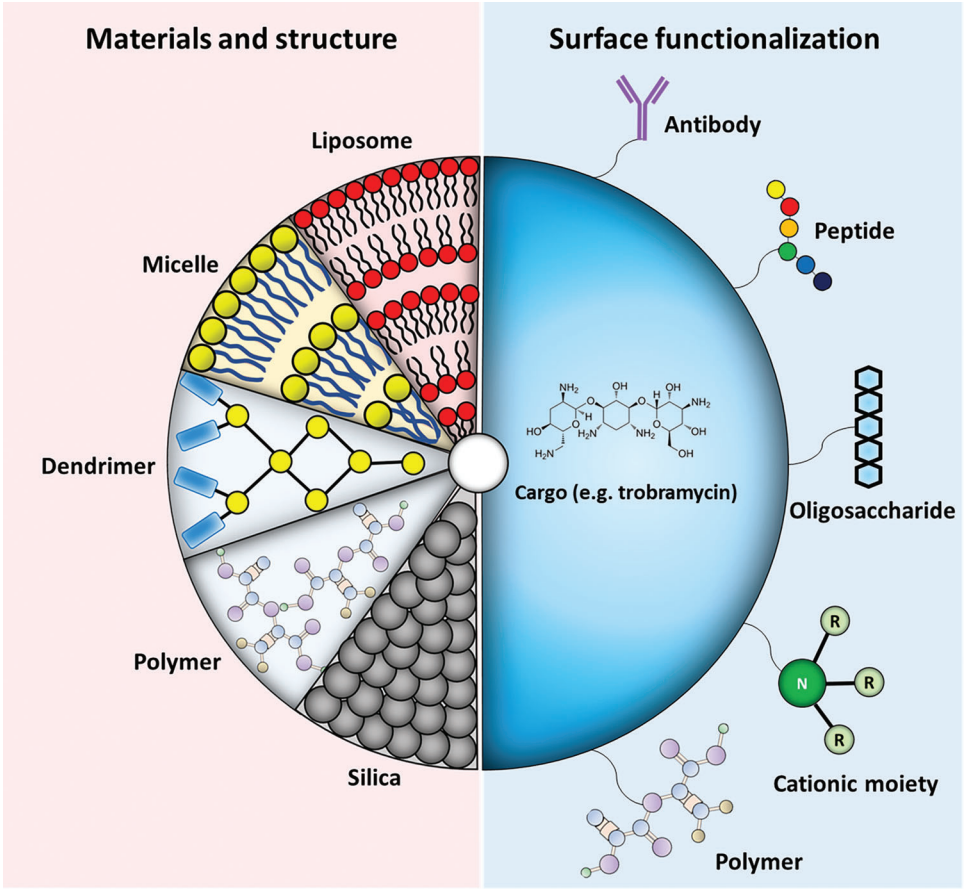

Figure 6. Nanocarriers can be made of different structures and materials (e.g., polymers, micelles, liposomes, inorganic materials) and functionalized on their surface (e.g., with antibodies, lectin-binding oligosaccharides, peptides, cationic moieties, etc.,) for targeted delivery of anti-infective cargos (e.g., tobramycin as shown here) across bacterial barriers.

materials can also increase the stability of a drug carrier to deliver other anti-infective molecules.[105,121]

A recent study showed that it is possible to harness the natural machinery found in *Photorhabdus asymbiotica* to inject macromolecules into cells. Although this was so far demonstrated for the delivery of CRISPR-CAS9 into mouse cells, such an approach would have enormous potential for anti-infective therapies if it could be translated also to bacteria.[122]

2.2.3. Exploitation of Specific Transport Pathways (Trojan Horse Approach)

The Trojan horse approach refers to conjugating one or more antimicrobials to a carrier molecule to achieve uptake by bacteria in a camouflaged manner via nutrient transporters. The drug either exhibits its action in the conjugated form or is released from the carrier molecule to become active. The transporters should be expressed mainly in bacteria with no or little expression in mammalian cells.

The most investigated transporters for this approach are the Ton-B-dependent transporters by utilizing siderophores as the carrier molecules. Researchers could have known approved antibiotics conjugated to native or artificial siderophores and show increased activity. The same approach is being further investigated by targeting the bacterio-specific siderophore receptor FepA.[123,124] Recently, another research could conjugate siderophores to TonB box peptides to allow better uptake into the bacteria, resulting in a superior antimicrobial activity in iron-limited growth media.[125] However, conjugating drugs with siderophores comes also with some drawbacks. Most importantly, bacteria can develop resistance by down-expressing the transporter leading to reduced uptake of the conjugate in the first place. A handful of molecules have been tested preclinically and clinically in this regard such as pirazmonam (Squibb), U-78608 (Upjohn), SMC-3176 (Astra-Zeneca), BAL30072 (Basilea), and cefiderocol (Shionogi). For the majority of them, the components of the iron transporter system got mutated or downregulated. This was avoided in the case of BAL30072 when using iron-limited media.[126] The latter may be indeed relevant for clinical settings like urinary tract infections due to low levels of iron in urine. Nevertheless, resistance is likely to develop elsewhere in the human body and render the potential antibiotic inactive.[127] Furthermore, cefiderocol was approved as a drug product in 2020 by both EMA and FDA.[128,129]

Alternatively, the Trojan Horse approach can also be implemented for passive transporters like the, e.g., LamB channel for maltose. Researchers have conjugated trimethoprim to maltose with a disulfide bridge as a release mechanism and proved that this leads to better accumulation inside the bacteria. However, they failed to demonstrate the superiority of the conjugate to free trimethoprim in terms of antibacterial activity that is expected because trimethoprim is a small molecule and permeability is not the limiter for its activity.[130] Additionally, others used a maltotriose conjugate, which was validated to be taken up by bacteria using the LamB channel.[131]

2.3. Infection-Triggered Drug Delivery

A promising strategy to selectively deliver potent anti-infectives to their bacterial targets is by conjugating the active agent to moieties that can only be bio-degraded by microenvironmental conditions related to the infection or under specific biochemical stimuli to release the drug. Most relevant strategies rely on materials that can be cleaved in the acidic pH found at the infection site or by an enzyme that is secreted either by the pathogen or the host as a response to the infection. The key to accomplishing this is a very good understanding of disease-related pathology and microbiology as well as the provoked microenvironment.

Bacterial-selective targeting was achieved by conjugating the antibiotic colistin to a modified fragment of the human AMP (ubiquicidin) by introducing a linker that is cleaved at infection sites by neutrophil elastase to release colistin.[132] Similarly, a desferrioxamine B-ciprofloxacin conjugate with "trimethyl-lock"-based linkers designed to release the antibiotic after exposure to bacterial esterase was reported to exhibit good antimicrobial activity against different bacterial strains.[133] Bacterial enzymes secreted into the biofilm matrix can also trigger drug release. Enzymatic release via bacterial gelatinase and hyaluronidase was also achieved by doxycycline-loaded core-shell nanoparticles. The gelatin core was surrounded by a double coating of CS and hyaluronic acid from the inside out. The nanoparticles were applied to an in vitro and ex vivo wound infection model of *Vibrio vulnificus* biofilms. Upon exposure, the outermost shell layer composed of hyaluronic acid was degraded by hyaluronidase present in the EPS. The underlying CS layer enhanced the penetration and retention of the nanoparticles into the EPS. Eventually, swelling of the CS core increased access of bacterial gelatinase to the gelatin causing subsequent core degradation and drug release leading to high biofilm penetration and eradication efficacy in comparison to the free drug.[134]

The concept of infection-triggered drug delivery can also be synergistically combined with adjuvanted carrier molecules. A pH-responsive polymer-drug conjugate made of a biodegradable cationic polymer HEX-Cys-DET and streptomycin was designed for this purpose.[135] The conjugate is neutral under normal physiological conditions but becomes positively charged in infected tissues with low pH, resulting in antibiotic release as well as enhanced activity of streptomycin because the polymer can induce pores in the bacterial membrane that improves the transport of the antibiotic into the bacteria. In addition to its effect on planktonic bacteria, the conjugate was found to effectively penetrate bacterial biofilms as well as being taken up by mammalian cells that might be needed to combat intracellular infections. Subsequently, this supports the conclusion that acidic pH in biofilms can be utilized for the concept of infection-triggered release. In this regard, cationic farnesol-loaded nanoparticles were formulated to have the ability to retain at the infection site for longer times due to their affinity to biofilm and pellicle. The pH-responsive core, made of 2-(di-methylamino)ethyl methacrylate, butyl methacrylate, and 2-propylacrylic acid, released the loaded drug inside the biofilm. This led to an 80% reduction of the biomass of S. mutants in vitro and attenuate the number and severity of carious lesions in vivo. This system was further modified and enhanced by the same research group to achieve a higher acid sensitivity and enhance the biofilm

reduction capacity.[136–138] Responsiveness to pH is one of the most frequently used triggering strategies. However, this concept can also be implemented to deliver non-drug species. For instance, nanoparticles were designed to release radicals in the proximity of the bacteria to allow for membrane destruction and thus bacterial killing at very low concentrations.[139]

Infection-triggered drug release can be combined with biofilm penetrating approaches to further enhance biofilm eradication and decrease off-target effects. To combat chronic lung infections, azithromycin-conjugated nanoparticles were produced with the ability to form size and charge adaptive clusters. These clusters are negatively charged in physiological pH enabling long circulation and accumulation in infected lung tissues. However, in acidic microenvironments as found in biofilms, these NP clusters can disassemble and release azithromycin-conjugated PAMAM nanoparticles. This conversion is accompanied by a size decrease from 112 to 6.5 nm and a charge reversal from −2.2 to +23.8 mV. The released nanoparticles with small size and positive surface charge exhibited excellent penetration and retention capabilities accompanied by an antibacterial effect against *P. aeruginosa* biofilms in vitro and in vivo.[140] Intensive research in the field of infection-triggered drug delivery will not necessarily require the development of novel anti-infective molecules. By boosting their antimicrobial activity and reducing undesired off-target effects it may even be possible to improve the potency of existing anti-infectives that could not yet reach clinical applications so far.

3. Advanced In Vitro Models for Developing Anti-Infective Therapies, Especially by Improving their Delivery

The need for novel anti-infectives and the problem of AMR has been well recognized by the scientific community and already led to many novel concepts and approaches in the last decade. Nevertheless, to facilitate the translation from bench to bed, predictive infection models for the preclinical development of such drug products are a necessity. Similar to the development of new anti-infective molecules, the development of novel drug carriers and formulations requires testing for both safety and efficacy. For the purpose of this perspective, we will discuss in particular preclinical models with a focus on pulmonary infections and drug delivery.

Animal models are considered essential, mainly because of the necessity of investigating immune responses as well as host-pathogen interactions. For many years, the known physiological differences between animals and humans in addition to their different responses and sensitivity to human pathogens have raised great concerns regarding the predictivity of such animal models for clinical outcomes.[141,142] Furthermore, the use of in vivo models for experimental reasons is restricted by legal regulations because of ethical concerns.[143] Each project that includes the use of animal models must be evaluated by ethics committees to confirm that it follows the "3R-rule": Replace, Reduce, and Refine.[144]

Animal models for chronic infections implicate an additional challenge due to the timelapse needed to develop the infection.[145] For instance, a classic model to mimic chronic *P. aeruginosa* lung infections starts with an inoculum of bacteria embedded in agarose or alginate beads to prolong the survival of the animal and hence the life span of the experiment.[146] Apart from the open question of whether the biofilm is really growing on the pulmonary mucosa rather than only in the agar beads, this model is problematic, because some of the animals do not even get sick while others die immediately, and only a small fraction of the animals develop symptoms that can be used to measure the anti-infective effect of a given treatment. Moreover, for this group of animals, the course of the experiment is extremely painful.

All these concerns, drawbacks, and restrictions necessitate the use and development of alternative methods to animal testing. An important milestone in this field is probably the recent *FDA Modernization Act 2.0*.[147] By replacing the former law from 1938, animal experiments are no longer mandatory for testing new drugs before entering clinical trials. This development already has and will still further stimulate the research on alternative models. Those are typically based on advanced in vitro techniques like, e.g., native or reconstituted tissues, novel cell lines, organs-on-chip, micro-physiological systems (MPS), and other CIVMs. In the future, in silico approaches like computer models, simulations and artificial intelligence will become increasingly important.

3.1. Biofilm Models

For modeling chronic infections, typically involving bacterial biofilms, there is no "gold standard" as each model may provide an answer to a specific question. In vivo models always implicate the suffering and killing of animals and at the same time are limited by (patho-)physiological differences to humans. In contrast, in vitro models may be based on human cells and tissues, allowing to reduce the complexity of a living organism to the biological factors of interest. Making reliable observations in a controlled environment is essential for the necessary validation of any model – either in vivo or in vitro – to eventually predict clinical outcomes.

3.1.1. "Biofilm-Only" Models

The first biofilm models were based on bacteria seeded on plastic surfaces either under static conditions, e.g., microtiter plates, or dynamic conditions, e.g., flow cells.[148] Such relatively simple in vitro models offer high reproducibility, reliable read-outs, and a rather high throughput, which is essential in early drug development. However, the lack of any host factors or even host-pathogen interactions makes it difficult to draw further-going conclusions based on these studies. Moreover, such simple models are often based on laboratory bacterial strains like *P. aeruginosa* PAO1. The latter has the advantage of being rather robust and relatively safe to work with, but its mushroom-like structures are not observed in clinical situations. Assays with laboratory strains may therefore cause a technical bias and were found to be of limited relevance for the clinical situation, e.g., in cystic fibrosis patients [149] The problem with clinically relevant strains, on the other hand, is that they don't adhere well to plastic surfaces and are easily washed away in these experiments.[150]

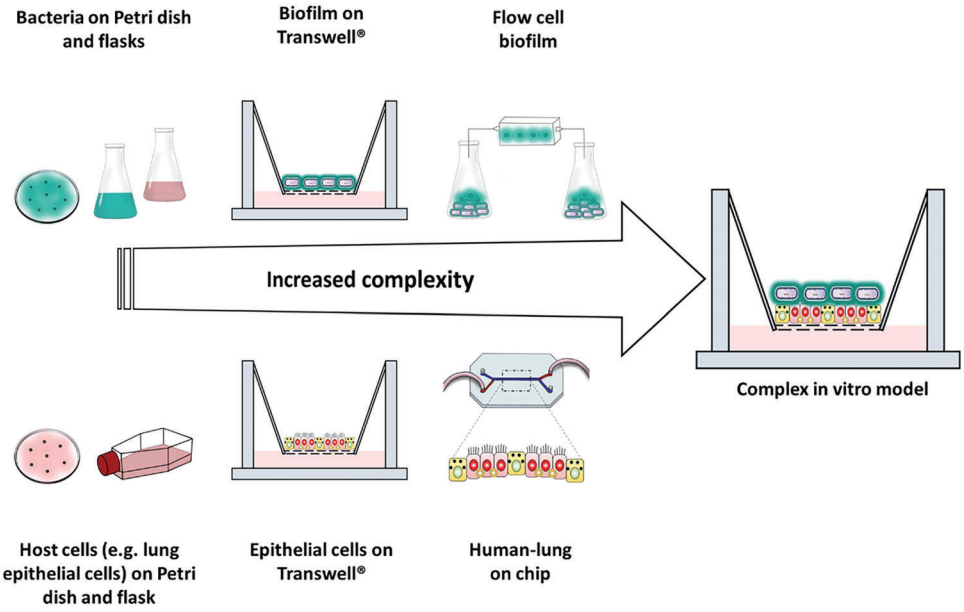

Figure 7. From simple to complex in vitro models (CIVMs) for assessing the activity and safety of anti-infectives. Bacterial models (upper part) to test the activity span from early planktonic cultures to mature biofilms, grown in simple, static to complex microfluidic devices. Host models (lower part) start from simple single-cell cultures to more complex 2D/3D co-cultures of several cell types. Combining such bacterial and host models is not trivial but may eventually allow to generate read-outs for both the pathogen and the host cells, to better predict outcomes of clinical trials as alternative to animal models.

As the limitations of simple biofilm models have been realized by the scientific community, efforts have been made to make them more physiologic by adding some biological complexity. For instance, bacteria may be cultivated in an environment that contains relevant body liquids such as saliva or mucus or biorelevant surrogates thereof. In this regard, an artificial sputum medium with a composition that closely resembles the sputum of cystic fibrosis patients was developed.[151] This medium contains several biologically relevant components, like DNA and mucins, which allows the bacteria to form biofilms that are more comparable to those in vivo. Such media may be further optimized by addressing also rheological properties, leading to a pulmonary mucus surrogate that can be used to investigate antibiotic activity and permeation of biofilms.[152,153] However, despite such progress, these systems still do not yet address any cellular host-pathogen interactions.

3.1.2. Complex In Vitro Models Comprising Bacteria and Host Cells

The restrictions of animal models on the one hand and the limitations of simple in vitro models on the other hand form a substantial bottleneck for the development of novel anti-infectives. So-called CIVMs may provide a perspective to close this gap.[3] For instance, mixed cultures of human cells and biofilm-forming bacteria could be used as a platform for drug testing, allowing to generate readouts of the host cells in parallel to monitoring bacterial responses (**Figure 7**). The main obstacle to achieving this is the time-dependent formation of bacterial biofilms. Not surprisingly, first approaches led to the insight that combining bacterial and eukaryotic cells causes rapid death of the latter during the time frame of biofilm formation.[154] On the other hand, the presence of human epithelial cells may lead to an alteration of bacterial behavior and in particular their antibiotic susceptibility.[155] To solve this obstacle, researchers have implemented advanced cell culture techniques, like the rotating-wall vessel, to create 3D cell aggregates for maintaining good viability over weeks under normal culture conditions. Nevertheless, 3D-aggregates of human alveolar A549 cells were only stable for 6 h upon infection with *P. aeruginosa*, and probably suitable for investigating the onset of acute infections, but not the further time course or even chronic infections.[156] Others investigated the direct interactions between *P. aeruginosa* and bronchial epithelial cells using both static and flow cell culture approaches, but were only able to study the interactions with early biofilms in the time frame of their experimental set-up.[157]

It may be hypothesized that an ideal system to study chronic infections in vitro would involve exposing the mammalian cells to pre-maturated biofilms instead of aiming for biofilm formation in co-existence with intact and viable mammalian cells. For this, mature *P. aeruginosa* biofilms were grown on 96-peg-lid inserts and then transferred to a standard 96-well microplate that contains A549 lung epithelial cells. Bacteria can eventually immigrate from the pig inserts and interact with the epithelial cells that allows investigation of the alterations that happen to the epithelial cells on the metabolic and genomic levels upon exposure to either planktonic bacteria or bacteria dispersing from mature biofilms. However, this model consists of bacterial biofilms and mammalian cells in the same compartment, but not in direct contact with each other. The lack of direct contact between both mammalian and bacterial cells in addition to some other technical restrictions limits the use of this system for drug testing.[158]

Following up on this approach and bearing in mind the importance of host-pathogen cross-talk for biologically relevant drug

testing, planktonic *P. aeruginosa* bacteria were first seeded in standard well plates until they had formed mature biofilms. Subsequently, micro clusters of this biofilm were transferred on the air-liquid-interface (ALI) of separately grown, tight epithelial cell monolayers. This co-culture of human-pulmonary epithelial cells and mature bacterial biofilms on Transwell inserts could be maintained for up to three days to perform relevant drug testing and to generate appropriate read-outs for both the host end and the bacteria. For instance, it was possible to monitor epithelial cell viability, transepithelial resistance (TEER), and cytokine release but also the number of colony-forming units (CFU) and the release of virulence factors for the bacteria.[159] Currently, this approach is being further improved by implementing 3D-bioprinting of bacterial biofilms on top of human epithelial cells instead of pipetting.[160] By eventually allowing to study chronic lung infections more reliably than with the existing animal models, such CIVMs could close an important gap in the pre-clinical drug development of novel anti-infectives, as displayed in Figure 7.

3.2. Models to Quantify Transport Across the Bacterial Cell Envelope

The bacterial cell envelope is quite complex and hard to mimic or understand due to its interfering components like lipids fused with polysaccharides, and some very specific proteins embedded in between, as illustrated in Figure 4. This biological barrier is also dynamic, as the bacteria can up- or down-express some proteins in response to environmental changes. The purpose of such a complex system is to acquire nutrients inside the bacterium needed for growth and survival, as well as efflux systems to secrete waste products. Evolution has made bacteria selective on what to take up and what not to a surprising extent. A variety of tools can be used to determine the mechanism of uptake for molecules like co-crystallography or in silico docking experiments, but this is technically demanding and still not always accurate. To develop new therapeutics, simple but predictive models would be most desirable to distinguish different anti-infectives by their potential and thus to predict bacterial bioavailability.

3.2.1. Predicting Bacterial Bioavailability

For most modern anti-infectives, designed to avoid the problem of AMR, the site of action is inside the bacterium. For those compounds, bacterial bioavailability in vivo is the product of their pharmacokinetics in the human body, permeability into the bacterium, efflux, and possible metabolism. Like for any other drug, the resulting concentration time course of an antibiotic at the site of action is decisive for its antimicrobial activity. Realizing this, researchers have developed many tools to predict or measure the intracellular concentration of the drug. High throughput screening allowed to predict patterns that determine the bacterial accumulation of small organic molecules. These early assays could not distinguish between the portion of the drug stuck to the outer membrane and the portion accumulated intracellularly, but this obstacle was solved over time. Small organic molecules are mostly internalized by the porins of bacteria, and this explains the factors that need to be considered for their uptake (e.g., amines are amphiphilic, rigid, and have low globularity that facilitates their uptake). Understanding this allowed a better design of compounds to optimize bacterial bioavailability. However, care must be taken that such modifications may not affect the affinity to the drug's intracellular targets leading to other limitations.[161,162]

In contrast to small organic molecules, bigger molecules (e.g., peptides, oligomers, polymers) interact with various parts of the bacterial membrane, which makes it technically challenging to investigate the precise mechanisms. For these reasons, models have been proposed to study different parts of the bacterial cell envelope separately (LPS, periplasm, inner membrane) and to combine such data with subcellular accumulation to understand the interplay of the bacterial components.[161,163,164] The accumulation pattern, e.g., preferentially in the periplasm or the cytoplasm, may help to elucidate the mechanism by which compounds are crossing the membrane.[11]

Some potential for drug discovery and delivery lies within the design of molecules that can pass through the bacterial membrane, but not by following the typical transport through the porins. Interesting compounds also include relatively high molecular weight molecules (>600 Daltons). For designing these molecules, researchers need to exclude porins from the equation and thus need porin-free models that will be discussed in the upcoming section.

3.2.2. Bacterio-Mimetic Envelope Models

Bacterial assays that involve testing directly against bacterial populations or colonies are the most useful first tests for indicating the antimicrobial activity of a given drug molecule. However, such end-point assays do not measure or explain bacterial uptake. Obtaining this knowledge could be the key to overcoming the obstacle of bacterial resistance by modifying the already existing antimicrobials to change their uptake mechanisms and make them more effective. To reach such outcome, different components of bacteria responsible for uptake, should be isolated and investigated.

Efforts have already been made to establish different types of in vitro non-cellular bacterial cell envelop models, including bacterio-mimetic vesicles, which are made of lipids, LPS, and proteins to mimic the bacterial envelope,[161,163] and so-called bacterial ghosts, which are empty bacterial membranes after killing the bacteria.[165] In our labs, an approach was introduced to reconstitute the bacterial cell envelope on Transwells by mixtures of bacterial lipids, LPS, and/or proteins, allowing to conduct such transport studies relatively fast and at high throughput. Such assays can also be customized according to the suspected mechanism of transport by varying the lipid composition (e.g., with and without LPS) or using porin-mimetic hydrogels. Transwells may also be coated with outer membrane vesicles to better mimic the bacterial membrane. Although still under development, such tools may help to figure out by what rate and extent and by what pathway a molecule is taken up into a bacterium.[161,166,167]

3.2.3. Computational in Silico Approaches

Wet lab experiments are always needed to generate actual data on the permeation of different molecules through the bacterial cell envelope. Nevertheless, the interest in predicting these processes through computational models has been growing. These in silico models are more cost-effective and may provide even more data. Essentially, however, such predictions must be validated against data generated by wet-lab assays in bacteria and in vitro. For instance, researchers have tried to build models of how porins may allow molecules to pass through by molecular dynamics and found their data in agreement with bacterial whole-cell assays.[168] Others were able to successfully predict what happens on the molecular level when ciprofloxacin passes through OmpC.[169] However, at present these models still lack accurate head-to-head comparisons due to the potential bias in the programmed experimental parameters, which can differ from the realistic parameters. Nevertheless, in silico models are already beneficial to answer pre-preliminary questions like whether a molecule can use this uptake pathway or not and estimate its potential at a very early stage of development.

To simulate the permeation of larger molecules through bacterial membranes by molecular dynamics, all-atom or coarse-grain models could be implemented, where researchers may be able to predict how membrane lipids interact with molecules on the surface or penetrate through the lipids. Such knowledge is helpful to determine that moieties are essential and to understand their impact on membrane permeability. In a first paper, the ranking of the permeability of some compounds was in correct agreement with the in bacterio data, also allowing the explanation of the uptake mechanism in a very detailed manner.[164] In another study, it was possible to determine the role of different amino acids in the permeation of the AMP pleurocidin through the membrane lipids of the bacteria, especially that amino acids were responsible for the first contact and embedding into the lipids, which is very useful for designing efficient AMPs.[170]

Computational in silico approaches may offer attractive high throughput and speed, but remain limited in terms of accuracy because they can only be as good as the data and parameters integrated into the underlying algorithms. Nevertheless, these models can be continuously enhanced by training the algorithms to predict that molecular descriptors yield the most influence regarding the uptake of molecules. This requires adequate training and test sets based on molecules previously characterized in bacterio or in vitro.

3.3. Bacterial Metabolism as an Emerging Barrier

Despite the progress in understanding and modeling the barrier function of biofilms and the bacterial cell envelope, bacteria also express metabolic enzymes that may alter the structure and thus limit the antimicrobial activity of antibiotics.[10] A very prominent example of such enzymes is the ß-lactamases, which are known to limit the activity of penicillins and carbapenems for many years.[171] Otherwise, however, our knowledge of bacterial metabolism and its role as a delimiter of bacterial bioavailability is still in its infancy. With the emergence of AMR, it may be well conceivable that bacteria develop novel, hitherto unknown metabolic enzymes that may be well relevant as biological barriers to anti-infective molecules.[172] Developing models that can distinguish and predict the role of metabolic enzymes would be helpful in this context. Data obtained from in vivo studies are not conclusive because of their complexity and the possible involvement of other biological barriers, such as the bacterial cell envelope, efflux pumps, and the biofilm matrix. To address the role of bacterial metabolism more specifically, an obvious approach would be to first isolate and purify bacterial lysates containing potentially anti-infective degrading enzymes. Like in metabolic studies with mammalian cell preparations, e.g., liver cells or mitochondria, such data must be interpreted with caution, because enzymes acquired after bacterial lysis might lose their functionality during preparation.[173] In a second step, degradation studies may be carried out to quantify the activity of the enzymes toward the anti-infective molecules in question in order to optimize their structure or to design protective materials and delivery systems. Once a satisfactory amount of in vitro data has been generated, it may be possible to establish structure-stability relationships and implement timely artificial intelligence and machine learning approaches. For the time being, however, some in vivo experiments will also be needed to demonstrate the relevance of such a concept in a more complex biological environment.

4. Summary and Outlook

In this perspective, we have described a strategy for improving the delivery of anti-infectives, based on the concept of bacterial bioavailability. The latter is determined by drug permeability across the biofilm matrix and the bacterial cellular envelope, alongside elimination by either efflux or metabolism. Future anti-infective therapies, capable of coping with the emerging threat of AMR, require improving not only on active molecules but also on their delivery across the aforementioned biological barriers. For the development of better anti-infective drug delivery systems, novel functional materials are pivotal, while an array of predictive in vitro and in silico models would clearly facilitate this process.

To overcome the biological barriers of bacteria, novel pharmaceutical materials are desired with properties similar to pathoblockers, i.e., capable to change the behavior and the properties of pathogens, but not necessarily killing them. Besides small organic molecules based on natural products or chemical synthesis, also macromolecular biopharmaceuticals are about to appear as novel anti-infective modalities, e.g. peptides, proteins, and antibodies, as well as oligo- and polynucleotides, such as siRNA and mRNA, up to complex systems like CRISPR/CAS. Despite their enormous potential, those molecules do need some enabling delivery technologies even more than the small molecules for approval as safe and efficient medical products. At the same time, improving their delivery might also allow to repurpose some existing anti-infectives, for which success in the clinic was hitherto limited by insufficient bacterial bioavailability.

As regards the clinical translation of novel materials as excipients or carriers for antimicrobials, we are facing an unconventional situation. It is not that many years ago, that understanding the biological and physiochemical properties of bacterial barriers came in the focus of pharmaceutical research. In analogy, anticancer drugs have existed for decades, but their targeted delivery to tumors and across their barriers has much improved their

efficacy. With a better understanding of the biofilm matrix, the bacterial cell envelope, and bacterial metabolism, we might achieve similar improvements for anti-infective therapies. Regarding development time and costs, repurposing existing drugs for other indications as adjuvants to existing antibiotics is the fastest and easiest strategy as in the case of combining N-acetyl cysteine with antibiotics. As this will not solve all the problems, however, we definitely shall need some new materials to be used as excipients and carriers. Demonstrating their safety is costly and time-consuming, and pharmaceutical companies are typically reluctant to make such investments for so-called "excipients" rather than for some new anti-infective "cargos".

Overcoming the COVID pandemic and the fast success of mRNA-based vaccines was possible by repurposing already approved nanocarriers. But existing pharmaceutical materials and carrier systems like, e.g., poly-(lactic-co-glycolic) acid and solid lipid nanocarriers, will probably not suffice to address all the delivery problems of emerging antibacterial therapies. Novel delivery strategies, materials, and carriers, for which we have provided some examples in this perspective, require the dedication of significant time and effort for research and development to reach clinical trials.

To facilitate the translation of novel concepts for improved anti-infective drug delivery successfully to the clinic and eventually to marketed products, two major fields of research should be boosted: The first is on novel functional materials as needed to accomplish the emerging delivery needs. The second is on advanced infection models, which – in accordance with the FDA Modernization Act 2.0 – do no longer need to involve animal experiments, but also allow CIVMs and emerging in silico tools, to provide mechanistic insight, reduce attrition and accelerate translation to clinical trials and eventual regulatory approval.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

anti-infective drug delivery, bacterial bioavailability, biofilms, biological barriers, chronic lung infections, complex in vitro models, gram-negative bacterial cell envelope

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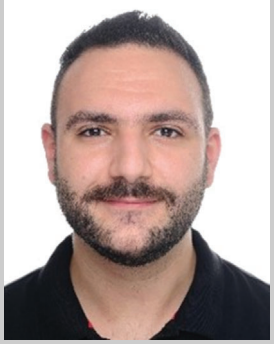

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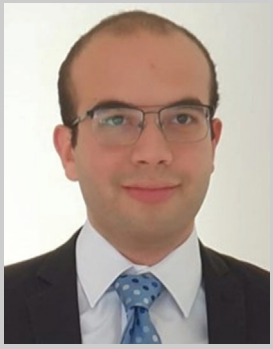

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