Anti-Infectives in Drug Delivery – Overcoming the Gram Negative Bacterial Cell Envelope

Florian Graef, Sarah Gordon and Claus-Michael Lehr

Abstract Infectious diseases are becoming a major menace to the state of health worldwide, with difficulties in effective treatment especially of nosocomial infections caused by Gram negative bacteria being increasingly reported. Inadequate permeation of anti-infectives into or across the Gram negative bacterial cell envelope, due to its intrinsic barrier function as well as barrier enhancement mediated by resistance mechanisms, can be identified as one of the major reasons for insufficient therapeutic effects. Several *in vitro, in silico* and *in cellulo* models are currently employed to increase knowledge of anti-infective transport processes into or across the bacterial cell envelope, however all such models exhibit drawbacks or have limitations with respect to the information they are able to provide. Thus, new approaches which allow for more comprehensive characterization of anti-infective permeation processes (and as such, would be usable as screening methods in early drug discovery and development) are desperately needed. Furthermore, delivery methods or technologies capable of enhancing anti-infective permeation into or across the bacterial cell envelope are required. In this respect, particle-based carrier systems have already been shown to provide the opportunity to overcome compound related difficulties and allow for targeted delivery. In addition, formulations combining efflux pump inhibitors or antimicrobial peptides with anti-infectives show promise in the restoration of antibiotic activity in resistant bacterial strains. Despite considerable progress in this field however, the design of carriers to specifically enhance transport across the bacterial envelope or to target difficult to treat (e.g. intracellular) infections remains an urgently needed area of improvement. What follows is a summary and evaluation of the state of the art of both bacterial permeation models and advanced anti-infective formulation strategies, together with an outlook for future directions in these fields.

F. Graef, S. Gordon
Department of Drug Delivery
Helmholtz Institute for Pharmaceutical Research Saarland (HIPS)
Helmholtz Center for Infection Research (HZI)
Saarbrücken, Germany
e-mail: Florian.Graef@helmholtz-hzi.de
Sarah.Gordon@helmholtz-hzi.de

C.-M. Lehr
Department of Drug Delivery
Helmholtz Institute for Pharmaceutical Research Saarland (HIPS)
Helmholtz Center for Infection Research (HZI) and
Department of Pharmacy, Saarland University
Saarbrücken, Germany
e-mail: Claus-Michael.Lehr@helmholtz-hzi.de

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1. Introduction

The effective treatment of infectious diseases by means of anti-infective drug therapies is currently associated with a significant and increasing level of difficulty. The incidence of nosocomial infections caused in particular by pathogenic bacteria is an indicator of this problem. In Germany alone 400 000 – 600 000 hospital-acquired, bacterial infections occur per year; 7500 – 15 000 of such cases are in fact lethal (Akademie der Wissenschaften and Deutsche Akademie der Naturforscher 2013). These statistics are mainly due to the increasing incidence of bacterial resistance to drug therapy, leading to a lack of sufficiently active anti-infective treatment options. Gram negative bacteria are particularly problematic in this respect: as an example, carbapenem-resistant-Enterobacteriaceae (CRE, for abbreviations see Table 1) are capable of evading the action of almost all currently-available antibiotics. This dire trend leads to the occurrence of nearly untreatable infections, with only two 'last resort' antibiotics available (tigecycline and colistin) - neither of which are effective in every patient (McKenna 2013). We are therefore faced with a major global challenge with respect to the successful treatment of Gram negative bacterial infections (Wellington et al. 2013).

While resistance to anti-infective drug therapies is without doubt the primary threat to effective infectious disease treatment, the evolution of resistance is compounded by a number of additional factors. Firstly, the successful delivery of anti-infectives to their site of action constitutes a challenging and complicated task, even in the case of a wild type bacterium. This is due to the fact that the bacterial cell envelope, especially that of Gram negative bacteria, works intrinsically as a complex and significant biological barrier to the effective delivery of anti-infective compounds and formulations (see section 2.1, Nelson et al. 2009). The occurrence of several resistance mechanisms such as up-regulation of efflux pump expression, down-regulation or alteration of the expression of transport and channel-forming proteins (e.g. porins) and the production of enzymes (e.g. ß-lactamase) within this envelope structure therefore acts to compound an already existing problem for anti-infectives which must penetrate into or entirely through the bacterial envelope in order to reach their site of action (Dever and Dermody 1991). As a further factor for consideration, from the so-called golden age of antibiotic discovery - lasting from the 1950s to the 1960s (Fischbach and Walsh 2009) - until the introduction of the oxazolidinones in 2000, no new anti-infective class was able to successfully reach the market. This low flow within the antibiotic development pipeline continues today, meaning that the diminishing pool of effective therapies is not being replenished by newly-emerging treatment options.
The above described factors contributing towards the problematic nature of effective infection treatment can collectively be regarded as symptoms of a bacterial bioavailability problem. Such a bioavailability issue draws attention to two significant necessities in the area of anti-infective research.

The first is the desperate need for new models and strategies to better investigate and characterize the trafficking of anti-infectives into or across the bacterial cell envelope, in order to increase collective knowledge of the envelope as a barrier which needs to be overcome. As a second need, novel anti-infective candidates with new modes of action are required, as are new delivery strategies which enable effective penetration into or across the Gram negative bacterial cell envelope. The current document will attempt to address aspects of both research needs, outlining the state of the art in each area as well as potential or actual future research directions. Specific emphasis will continue to be given to Gram negative bacteria as particularly problematic pathogens.

Table 1. Abbreviations, in alphabetical order.

AMPs	Antimicrobial peptides
CRE	Carbapenem-resistant Enterobacteriacae
DUV	Deep ultraviolet
EPIs	Efflux pump inhibitors
IM	Inner membrane
LB	Langmuir-Blodgett
LS	Langmuir-Schaefer
LPS	Lipopolysaccharides
MD	Molecular dynamics
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
OD	Optical density
OM	Outer membrane
OMPs	Outer membrane proteins
PaßN	Phenylalanin arginyl ß-naphtylamine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PL	Phospholipid
PS	Periplasmic space
QSARs	Quantitative structure-activity relationships
QSI	Quorum sensing inhibitor
RND	Resistance-nodulation-cell division
SLBs	Supported lipid bilayers
SLNs	Solid lipid nanoparticles

2. The Gram Negative Bacterial Cell Envelope as a Bioavailability Barrier to Anti-Infectives

As already mentioned, the intrinsic structure of the Gram negative bacterial cell envelope presents a significant barrier to the successful delivery of anti-infectives. Therefore a brief overview of the major structural components of the cell envelope, including details of envelope modifications responsible for the occurrence and evolution of resistance, is first given here.

2.1 The Intrinsic Bacterial Barrier

The Gram negative bacterial cell envelope can be divided into three major parts, each of which constitutes a significant obstacle to anti-infective penetration (Figure 1). Starting from the bacterial cytoplasm and proceeding outwards, the inner membrane (IM) represents the first layer of the envelope barrier. It consists of a phospholipid (PL) bilayer mainly composed of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin, with incorporated transmembrane proteins and lipoproteins. The periplasmic space (PS) with the peptidoglycan cell wall constitutes the second layer. Peptidoglycan, a polymer of repeating disaccharides, is responsible for the maintenance of cell shape and structure. The surrounding area is a highly viscous, aqueous compartment, densely packed with proteins (Mullineaux et al. 2006). Furthermore defense mechanisms including enzymes (e.g. ß-lactamase) are also located within this space. The outer membrane (OM) forms the third envelope sub-structure. The membrane itself is asymmetric in nature, being subdivided into a PL- (mainly PE) containing inner leaflet, and an outer leaflet mainly consisting of lipopolysaccharide (LPS). LPS in turn is composed of the so-called Lipid A, a phosphorylated glucosamine with six to seven acyl chains which anchors LPS to the inner leaflet of the OM; a core oligosaccharide; and the outermost portion of the molecule, the O-antigen. LPS acyl chains are mainly saturated, which confers a gel-like structure on the molecule. Association of gel-like LPS molecules within the outer leaflet is additionally strengthened by the presence of divalent cations being present in the surrounding medium, which neutralize the negative charge of LPS phosphate groups. This further contributes to the formation of a viscous structure which limits the permeation of hydrophobic compounds, including many anti-infectives and detergents. The OM also incorporates outer membrane proteins (OMPs) such as the porins (e.g. OmpF), which span the entire OM. Porins allow for and control the passive diffusion of hydrophilic compounds, for example ß-lactam antibiotics, with a size limit for such permeation of approximately 700 Dalton (Silhavy et al. 2010). The combination of

LPS and porins is therefore responsible for the strong permeability limiting properties of the OM, which acts to restrict the permeation of hydrophobic as well as hydrophilic compounds.

In addition, efflux transporters, most commonly belonging to the resistance-nodulation-cell division (RND) superfamily, feature prominently within the cell envelope. Sub-structures of these transporters are present in each of the three major envelope subsections (for example AcrB-AcrA-TolC and MexB-MexA-OprM where subunits are located in the IM-PS-OM) meaning that the pump as a whole spans the entire envelope structure. Such efflux pumps are responsible for the active excretion of compounds (e.g. anti-infectives) in an energy-dependent manner (Kumar and Schweizer 2005).

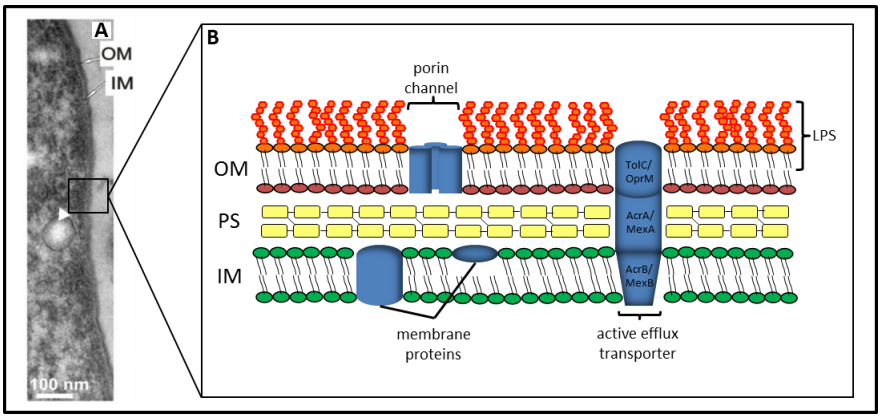

Fig. 1. Transmission electron microscopy image of the cell envelope of *Legionella dumoffii* (A, adapted from Palusinska-Szysz et al. 2012) and schematic overview of the Gram negative bacterial cell envelope, highlighting the most important structural components (B): the inner membrane (IM) incorporating transmembrane and lipoproteins, the periplasmic space (PS) housing the peptidoglycan cell wall, and the asymmetric outer membrane (OM) with its lipopolysaccharide (LPS) outer leaflet and porins. The general structure of an efflux transporter is also shown.

2.2 The Role of the Envelope in Mediating Resistance Mechanisms

In principle we can differentiate between three major, antimicrobial resistance strategies of bacteria: i) degradation of anti-infective compounds by bacterial enzymes, ii) protection of anti-infective targets by e.g. structural or expression modification, and, of most relevance to the current document, iii) alteration of the cell envelope barrier function (Davin-Regli et al. 2008), which will here be further described. Modifications to barrier properties result in an increased efflux in combination with a reduced uptake of anti-infectives, leading to inadequate intracellular anti-infective levels. The increased efflux of anti-infectives occurs due to an overexpression of efflux pumps (as mentioned above), which have a broad range of action and as such, are able to mediate resistance to a variety of anti-infective classes (Tenover 2006). Resistance in the context of reduced uptake arises due to bacterial modification of OMP copy numbers or conformation, and/or alterations in LPS structure. The expression of OMPs, in particular porins, can be down-regulated within the OM structure, or can alternatively be completely abrogated (Nikaido and Rosenberg 1981). The latter case is for example known from *Escherichia coli* isolates, which are resistant against cefoxitin due to an absence of the major OmpF porin channel (Tenover 2006). Furthermore, bacteria can modify the structure of their porins as a strategy to prevent anti-infective entry. Such structural modification can for example consist of a narrowing of the porin channel, which decreases the permeation of larger, hydrophilic compounds (De et al. 2001). The structure of LPS molecules can additionally be altered, in order to facilitate an increase in the barrier properties of the OM. The most effective mechanism by which LPS alteration leads to increased barrier function is via a reduction of negative net charge, leading to a reduced permeation of cationic anti-infectives (Kumar and Schweizer 2005).

2.3 Implications for Anti-Infective Drug Delivery

Clearly, the unique structure of the Gram negative bacterial cell envelope, together with the ability of bacteria to alter the structure and resulting functional activity of various envelope components, creates a considerable hurdle to the cellular permeation of anti-infectives. The development and application of models in order to facilitate an increased understanding of envelope permeation processes as well as the investigation of new anti-infective delivery approaches are therefore introduced and discussed below, as two research strategies required in order to address the issue of inadequate anti-infective permeation.

3. Strategies to Combat Intrinsic Difficulties/Bacterial Resistance Mechanisms Related to Anti-Infective Transport

3.1 Models for Characterization of Drug Transport Across the Bacterial Cell Envelope

As detailed above, the Gram negative bacterial cell envelope works as an effective biological barrier to the successful delivery of anti-infectives to their target site. The fundamental existing barrier properties of the envelope are also able to be further increased through the up-regulation of resistance mechanisms. Therefore, in addition to well-established and commonly used efficacy testing approaches, it is of considerable interest to obtain a greater and more detailed level of knowledge regarding rate, extent and mechanisms of the processes by which anti-infectives permeate (actively or passively) across the envelope. Models which mimic the cell envelope and so enable provision of such information can thus help to facilitate the rational design of anti-infective agents, capable of overcoming intrinsic delivery difficulties/bacterial resistance mechanisms. Such models could additionally contribute useful information to early anti-infective drug discovery processes. The currently existing and employed models of the envelope structure, used in order to provide permeation and transport information, will be described in the following section. The needs which are unmet by these existing models will also be mentioned.

3.1.1 Electrophysiology Studies

Electrophysiological studies are applied to obtain information about the transport of anti-infectives through single porins. The principle of electrophysiology is based on the reconstitution of such channel forming proteins - mostly OmpF, as the main porin responsible for the passive OM permeation of many anti-infectives such as the ß-lactams and quinolones - into planar lipid bilayers (Figure 2A). Such bilayers mostly consist of phosphatidylcholine (PC), and are made for example by bursting porin-containing proteoliposomes across an aperture within a solid support (Kreir et al. 2008). An external voltage is then applied across the aperture-spanning membrane, which causes an ion flux through the inserted porin channel. The strength of the resulting current allows for the provision of information regarding the channel structure and its functional properties in a variety of experimental settings (e.g. ranges of salt concentration, pH). The technique is additionally able to be automated (Mach et al. 2008a) and can be further optimized for example by applying the porin-containing supported lipid bilayer system into glass nanopipets (Gornall et al. 2011). In addition to providing information on porin

structure and function, anti-infective passage kinetics through the bilayer-reconstituted porins can be studied by the use of high resolution ion-current fluctuation analysis (Pages et al. 2008). In general, the permeation of anti-infective compounds through porins is detected by a decrease in current due to an occlusion of the porin channel by the permeating compound. Electrophysiological studies therefore facilitate determination of the direct translocation of charged molecules through porin channels. They additionally allow for evaluation of the interaction of anti-infectives with the constriction zone of porins (the narrowest part of the porin channel, which mediates the size-wise exclusion of molecule permeation across the OM) in particular. The relative affinity of different anti-infective compounds for specific porins can also be elucidated using electrophysiological studies – for example, enrofloxacin has been shown to have the strongest recorded affinity for OmpF. Combining the information obtained from electrophysiology studies with molecular dynamic (MD) simulations enables the identification of the specific anti-infective pathway across porin channels (Nestorovich et al. 2002; Danelon et al. 2006; Mach et al. 2008b). This further contributes to understanding the occurrence of porin structure-related resistance mechanisms. Again taking the case of enrofloxacin, a relatively simple modification to the OmpF channel (a single-point mutation in the constriction zone) has been shown by such a combination electrophysiology-MD approach to lead to a drastic decrease in anti-infective translocation (Mahendran et al. 2010).

3.1.2 Liposome Based Assays

Assays utilizing liposomes as artificial membrane models are also employed to investigate permeabilization effects of anti-infectives, as well as the extent of anti-infective permeation into such model membranes. The existing liposome-based approaches can be basically differentiated into two major categories. On one hand the so-called liposome swelling assays or leakage studies must be mentioned. These approaches are based on uni- or multilamellar vesicles made of a single PL species (e.g. PC, PE) with or without incorporated porins (Nikaido and Rosenberg 1983), PL mixtures, or PL-LPS (rough (without O-antigen) or smooth LPS (with O-antigen)) mixtures, utilized in an attempt to mimic the OM components. Polymers or fluorescent dyes are further incorporated into the central aqueous compartment or within the bilayers of such liposomes. This provides for an indirect detection method for permeation of the analyzed anti-infective, by means of tracking changes in optical density (OD), or via fluorescence analysis. As an example of such a setup, the anti-infective of interest is mixed with polymer-containing liposome dispersions (which often also have inserted porins) under isosmotic conditions. If the anti-infective is not able to penetrate into the vesicles, the measured OD will remain unaltered. If however the anti-infective compound is able to permeate into the liposomes, a swelling of the vesicles occurs due to an influx of water, caused by the presence of an osmotic gradient as mediated by the permeating

anti-infective compound. Anti-infective permeation and liposome swelling can ultimately result in bursting of the liposomes, leading to a release of the incorporated polymer, which is then detectable as a decrease in OD (Figure 2B). Liposome swelling or leakage assays also facilitate study of direct membrane disrupting effects of proteins (e.g. lamB or surfactant protein A) and antimicrobial peptides (AMPs, e.g. aurein 1.2) on artificial membrane systems (Luckey and Nikaido 1980; Kuzmenko et al. 2006; Fernandez et al. 2012; Fernandez et al. 2013). Of considerable current interest with respect to such assays is the use of liposomes which imitate more closely Gram negative bacterial membrane compositions. In this respect, liposomes made of LPS or PL-LPS mixtures as a more realistic OM mimic are also often used for swelling or leakage studies, or to investigate PL-LPS interactions within the model membrane. Such studies could help to improve understanding of the OM organization or modulation of the OM during the development of resistance mechanisms (D'Errico et al. 2009; Kubiak et al. 2011).

On the other hand, liposomes can be used to study the accumulation and uptake of anti-infective compounds in liposomal membranes by direct analysis of anti-infectives themselves. In this respect, anti-infectives of interest, which are either auto-fluorescent or fluorescently labeled, are incubated with liposomes. The relation between anti-infective structural characteristics/modifications and interaction with the artificial liposomal membrane can then be studied, as can anti-infective uptake into such model membranes. This is achieved by determining the accumulation of anti-infectives within the lipid membrane or their uptake into the vesicles, analyzed via nucleic magnetic resonance spectroscopy or fluorescence microscopy (Rodrigues et al. 2003; Ries et al. 2015).

3.1.3 Langmuir Trough Based Approaches

Mono- and/or bilayers prepared from PL and/or LPS in various combinations are also employed as models in anti-infective research. Such approaches facilitate investigation of the organization and interactions within artificial membrane systems and, with respect to permeation, study of the influence of antimicrobial proteins or peptides in particular on membrane integrity. In general, these experimental setups were originally developed to study interactions within mammalian-mimicking membrane structures, or interaction of external entities with such structures. Imitation of the double bilayer nature of the Gram negative bacterial envelope, as well as the OM, with particular emphasis being placed on its structural components and asymmetric nature, is however of considerable interest in the current application of such approaches.

Preparation of lipid monolayers on the surface of water or buffer is achieved by using a Langmuir film balance or trough, whereas lipid bilayers are mostly pre-

pared as supported lipid bilayers (SLBs) on silicon - less often mica or gold - surfaces as solid supports. SLBs in turn can be prepared from lipid vesicles which are fused onto the solid support, via Langmuir-Blodgett (LB) or a combination of LB and Langmuir-Schaefer (LS) deposition techniques (Peetla et al. 2009). An advancement of SLBs resulting in the production of more bacteriomimetic models is represented by the additional incorporation of floating lipid bilayers. Production of such a model involves combining three vertical LB depositions with one horizontal LS deposition (Figure 2C), resulting in the formation of a lipid bilayer which floats at a distance of 2-3 nm from the supported lipid bilayer (Charitat et al. 1999; Fragneto et al. 2012). Langmuir-derived lipid bilayers are often prepared from PC or PC-PG mixtures (in order to mimic the negative charge of bacterial membranes) and used to study the membrane insertion potential of AMPs, as well as their disordering and transmembrane pore forming abilities. Results show a higher affinity and disruptive effect on models composed of negatively charged PLs (Fernandez et al. 2012; Fernandez et al. 2013). Langmuir-produced monolayers made of PG with or without incorporated OmpF have also been used to investigate the interaction of antibacterial proteins such as colicins with the OM, as well as their pathway across the OM (Clifton et al. 2012). As mentioned above, many such Langmuir-based models additionally take the impact of LPS as the major OM structural component into account. In one instance, stable Langmuir monolayers were prepared at the air-liquid interface using rough strain LPS. Such a model provided valuable information about LPS structure at the air-liquid interface, and therefore constitutes a further step to a more accurate model of the OM (Le Brun et al. 2013). In a further approach, a realistic mimic of the OM structure was prepared by combining a PC bilayer deposited via LB on a solid support (representing the inner OM leaflet) with an overlying rough strain LPS bilayer (outer OM leaflet), introduced via LS deposition. This model successfully mimics the asymmetric nature of the OM, and was first employed to describe the molecular mechanisms of the well-known OM destabilization effect occurring as a result of the removal of divalent cations from surrounding media (Clifton et al. 2015).

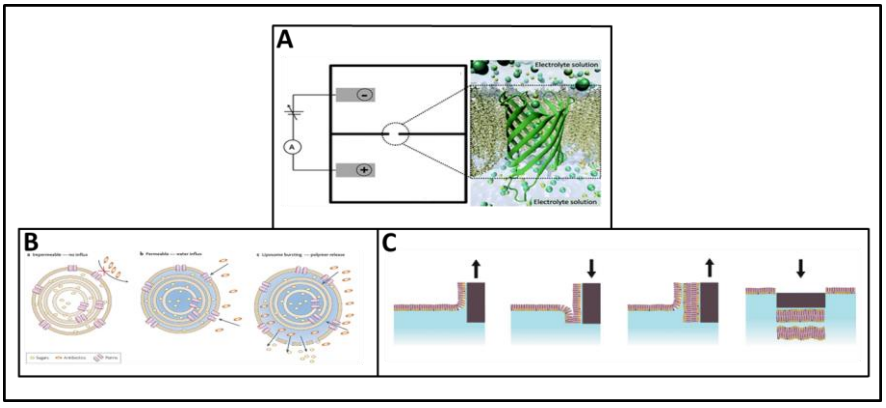

Fig. 2. Schematic overview of *in vitro* approaches to produce bacterial membrane models, for the characterization of drug transport. (A) The experimental setup for electrophysiology studies to investigate anti-infective transport through porins, incorporated in planar lipid bilayers (adapted from Modi et al. 2012 with permission from The Royal Society of Chemistry);(B) the principle of a liposome swelling assay, employed to assess permeabilization processes mediated by anti-infective compounds (reprinted from Pages et al. 2008 by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] Pages et al. 2008, copyright 2008); (C) the Langmuir-based preparation procedure for preparing floating lipid bilayers (from Fragneto et al. 2012, with kind permission from Springer Science+Business Media) is additionally depicted .

3.1.4 *In Silico* Methods

Besides the so-far described *in vitro* models, *in silico* approaches are also utilized to investigate the impact and interaction of various lipid species within simulated bilayers (which in turn may have a bearing on anti-infective permeability). They are furthermore applied to inform the development of membrane models which more closely and accurately reflect the structure and components of the IM and OM. *In silico* approaches may also be employed to determine the affinity and/or translocation of anti-infectives with or across porin channels, as alluded to previously (see 3.1.1). They may additionally be used to screen compounds for anti-infective activity based on quantitative structure-activity relationships (QSARs), defined via topological descriptors (numerical values correlating chemical properties with biological activity (Mayers 2009)) and physicochemical parameters. In one example, MD simulations have been used to mimic the IM – consisting of PE and PG in a 3:1 ratio, closely reflecting the *in cellulo* composition – in order to evaluate intra-bilayer PL interactions. Conducted simulations showed that interactions between these specific PLs are mainly based on H-bond formation and chiefly occur between PE and PG, less often between only PE molecules and almost never between PG molecules alone. As a consequence, the presence of PG within the membrane leads to a decrease in PE headgroup protrusion and a reduced mo-

tion along the artificial membrane; this results in an enhanced membrane stability, leading to a strengthening of the IM permeation barrier (Murzyn et al. 2005; Zhao et al. 2008). *In silico* studies which even more accurately reflect the PL composition of the IM have also been conducted. As a first step bilayers consisting of CL alone were simulated, to determine its biophysical role within membranes via evaluation of its charge-dependent lipid packing (Lemmin et al. 2013). Further, IM models which additionally include heterogeneous lipids, exhibiting different acyl chain lengths and cyclopropane rings, can be considered as yet further improvements towards an accurate IM mimic (Pandit and Klauda 2012).

The OM has also been simulated in various *in silico* studies, starting with models consisting of LPS alone and followed by simulations using a combination of a PL inner leaflet and LPS outer leaflet to more accurately reflect the asymmetric OM structure. These models have largely been used to study properties such as interactions between LPS molecules in the OM, the stabilization effect of divalent cations on the membrane structure (and resulting barrier properties), the effect of electroporation on the barrier function of protein-free, asymmetric membrane structures and the impact of OM enzymes as well as proteins on membrane integrity (Lins and Straatsma 2001; Wu et al. 2014; Piggot et al. 2011). Simulations of the OM as well as the IM have additionally been employed to study the interaction of AMPs with such artificial systems, highlighting the way in which AMPs are able to pass through and disrupt the bacterial envelope – firstly due to a self-promoted uptake across the OM, and subsequently as a result of disruption of the IM via the formation of micelle-like aggregates (Berglund et al. 2015). MD simulations have further been used to determine the molecular and rate-limiting interactions occurring during anti-infective permeation through porins on an atomic scale. Such studies allow for a better understanding of the translocation pathway and estimated permeation time of anti-infective compounds, as well as the way in which modifications in the porin channel constriction zone can affect and reduce anti-infective permeation (Mach et al. 2008b; Mahendran et al. 2010; Singh et al. 2012; Hajjar et al. 2010). *In silico* screening has furthermore been employed to define QSARs of anti-infectives by evaluating the impact of physicochemical properties such as lipophilicity and molecular weight on anti-infective activity (O'Shea and Moser 2008; Cronin et al. 2002). The definition of topological descriptors together with the performance of linear discriminant analysis further enable the attainment of discriminant functions, which allow for differentiation between active and non-active anti-infectives. Such functions can subsequently be applied to screen compound libraries for new lead structures showing promising anti-infective activity. (Murcia-Soler et al. 2003; Murcia-Soler et al. 2004).

3.1.5 *In Cellulo* Approaches

In cellulo approaches which give information about permeability processes by facilitating the determination of intra-bacterial accumulated anti-infectives are of enormous interest, as such approaches of course constitute the most accurate representation of the envelope structure. Within the scope of these approaches, a large number of bacteria are usually incubated with the anti-infective compound of interest. This is followed by washing to remove remaining extracellular and/or adherent compound, lysis of the bacterial cells and subsequent quantification of the amount of intracellular drug. LC-MS/MS methods are generally employed in order to quantify what often proves to be a very low level of anti-infective compound. Such quantification methods are also frequently applied to examine the permeation of various different anti-infectives tested on distinct bacterial strains (Cai et al. 2009; Davis et al. 2014). As such an approach is possibly error-prone due to the potential for inadequate removal of extracellular/adherent anti-infective, as well as the population-based rather than single-cell nature of the quantification process, approaches with direct single-cell resolution based on deep ultraviolet (DUV) fluorescence or the combination of a DUV fluorescence microscope with a synchrotron beamline have been employed. These approaches allow for quantifying fluorescent or fluorescently-labeled compounds, and for example have been used to compare anti-infective uptake in wildtype and mutant/resistant bacterial strains (Pages et al. 2013; Kascakova et al. 2012). It must be mentioned here however that such an approach is still limited to an 'inside/outside' distinction of anti-infective location, and determination of anti-infective permeation with any higher degree of spatial resolution remains extremely difficult.

3.1.6 Shortcomings of Existing Models and Future Directions

The current modeling approaches discussed in this section help to get a better understanding of permeation processes across various sub-structures of the Gram negative bacterial cell envelope. However, drawbacks and unmet needs can be mentioned for each of the above categories of models available to date. As a general comment, the *in vitro* and *in silico* modeling approaches described here mostly focus on producing or simulating structures which approximate either the IM or OM, and not the cell envelope as a whole – or, in the small number of cases where the overall envelope structure is approximated, the resulting model is often tailored to the examination of intra-membrane interactions or causes of membrane disruption. In addition, many such models consist of a phospholipid composition which deviates from that found *in cellulo*, and, while it has been mentioned that attempts are made in some cases to represent the asymmetric structure of the OM in models of this membrane component, many models still neglect to feature this important aspect. Furthermore, due to considerable difficulties associated with

scale and resolution, the vast majority of models to date allow for a qualitative prediction of anti-infective permeation and transport, rather than for quantification of such processes. *In cellulo* approaches where multiple planktonic cells rather than single cells are used have proven very useful in order to provide detailed and in some cases, quantitative insights into permeation processes; however, as mentioned, such methods generally rely on an average permeation within a bacterial population to draw conclusions regarding single cell permeation. Furthermore, current *in cellulo* approaches do not allow for evaluation of the specific extent of anti-infective permeation into the envelope structure. Hence, models which mimic the overall envelope in terms of their PL composition and structure, which are designed to explicitly investigate and quantify transport and permeation processes, and which are able to discriminate between active and passive permeation of anti-infective compounds and delivery systems in both a spatially- and kinetically- resolved manner are desperately needed.

3.2 Improving Bacterial Bioavailability using Advanced Delivery Strategies

In addition to employment and development of bacterial permeation models, a direct research focus is also placed on anti-infective therapies themselves in an attempt to overcome the cell envelope structure, achieve an increase in intrabacterial drug concentrations, and, in doing so, improve bioavailability in bacteria. In this respect, the search for new anti-infective drug candidates as well as the investigation of alternative approaches to antibiotic therapy continues, as presented and discussed in detail elsewhere. Additional strategies, such as the re-formulation of currently available anti-infectives with permeation-enhancing excipients or the application of advanced carrier systems, also represent promising research directions. Such strategies are particularly valuable in instances where bacterial bioavailability issues cannot be directly resolved by the introduction of new molecules, or through modification of existing anti-infective structures using medicinal chemistry approaches. As such, a number of currently investigated advanced formulation strategies are presented below.

3.2.1 Efflux Pump Inhibitors

As mentioned in section 2.1, efflux in wild-type as well as drug resistant Gram negative bacteria is mainly mediated by the RND superfamily of efflux transporters. The use of formulations incorporating efflux pump inhibitors (EPIs) which are able to interact with such pumps, decreasing anti-infective efflux and subsequently

leading to higher intracellular drug levels, therefore represents a useful strategy to restore anti-infective potency. The inhibition of pumps as mediated by EPIs can be described as occurring by two major modes of action. One can be classified as biological, in which EPIs act to decrease the expression of the pumps themselves by inhibiting transcription or translation via antisense oligonucleotides. A pharmacological mechanism represents the second mode of action, in which EPIs operate through direct interaction with the pump affinity site, acting for example to collapse the efflux energy or to competitively or non-competitively inhibit the efflux process (Van Bambeke et al. 2010). EPIs can be further differentiated into inhibitors with a narrow spectrum of activity, being used as diagnostic tools to detect active efflux, or inhibitors with a broad spectrum of action, which could be potentially useful in clinical settings. The further ability of EPIs to restore the activity of simultaneously applied anti-infectives (being visible for example in a decrease of minimum inhibitory concentration (MIC)) makes them an even more promising approach as a means to increase anti-infective bacterial bioavailability. Examples of known EPIs include analogues or lead structures of tetracyclines or fluoroquinolones, arylpiperidine and phenothiazine derivatives as well as peptidomimetics (Pages and Amaral 2009). Peptidomimetics with phenylalanine arginyl ß-naphthylamine (PAßN) as lead compound represent the first efflux inhibiting group which showed an effective blocking of fluoroquinolone efflux in a RND over-expressing strain of *Pseudomonas aeruginosa* (Renau et al. 2002). Currently, EPIs are used primarily as *in vitro* screening tools; their potential use in the clinic is still under investigation due to the existence of several challenging factors. The primary obstacle to the use of EPIs in a clinical setting is that of toxicity - most of the known EPIs to date need to be used in high concentrations, which may lead to possible toxic effects. Their use in combination with anti-infectives also demands the absence of interactions between the EPI and the anti-infective compound, as well as comparability in their pharmacokinetic profiles.

3.2.2 Antimicrobial Peptides

The use of the previously mentioned AMPs, either alone or especially in synergistic combinations with conventional anti-infectives, represents a further strategy to overcome anti-infective bioavailability problems by enhancing their transport across the bacterial cell envelope. AMPs can be further used as stimuli for the innate immune system, or as endotoxin-neutralizing agents (Gordon et al. 2005). AMPs in themselves are generally small cationic peptides which can be derived from humans, bacteria, or even viruses (Yount and Yeaman 2004). Their mode of action as bioavailability-potentiating agents is primarily based on the initiation of bacterial membrane perturbation, an effect mainly mediated by electrostatic interactions between the positively charged peptide and the negatively charged LPS of the OM. Such interactions lead to a destabilization of the OM by displacing present divalent cations, which facilitates penetration of AMPs and any other associ-

ated compounds through the OM structure. Following this self-promoted uptake through the OM, the further association of AMPs with the outer leaflet of the IM followed by the formation of micelle-like aggregates finally leads to a rupture of the bacterial envelope. This allows either for bacterial killing, or for an even further enhanced uptake of the simultaneously administered anti-infective. A non-destructive action of AMPs, facilitated by binding to DNA or RNA, is also further described (Hancock 1997; Hancock and Chapple 1999). Several studies report the potentiating effect of AMP–anti-infective combinations, resulting in an increased anti-infective activity even in hard to treat bacterial strains and biofilm forming species. In this respect, the synergistic effect of AMPs together with a wide range of anti-infectives with different modes of action could be demonstrated by the effective treatment of *Clostridium difficile* (Nuding et al. 2014). Furthermore, combinations of AMPs with anti-infectives have shown to result in an increased activity against the biofilm formation of Methicillin Resistant *Staphylococcus aureus* (MRSA), and have demonstrated a successful inhibition of *Pseudomonas fluorescens* (Mataraci and Dosler 2012; Naghmouchi et al. 2012). Hence, AMPs represent a promising approach to improve anti-infective bioavailability in Gram negative as well as Gram positive bacteria, as well as in particularly problematic bacterial infections involving biofilm formation. Several clinical trials, especially for topical application of AMPs to human subjects, are ongoing, but are associated with several challenges. In addition to the potential for toxic effects which could for example result from non-specific membrane disruption, the fast degradation and short half-life of AMPs constitute the main obstacles to generalized use (Park et al. 2011). The incorporation of AMPs into particulate carrier systems could potentially help to reduce or overcome these difficulties – such approaches are further discussed below.

3.2.3 Nanoparticulate Drug Carriers ("Nanopharmaceuticals")

Anti-infectives as free drugs may show low water-solubility, unfavorable pharmacokinetics, side effects or stability problems (Xiong et al. 2014) – all factors which intrinsically create problems for penetration into and effective action within bacteria. The incorporation of anti-infectives into carrier systems, such as liposomes, polymeric nanoparticles, solid lipid nanoparticles (SLNs) or dendrimers may help to reduce the impact of such characteristics, and as such presents several advantages compared to the use of free anti-infectives. In light of their typical size range, these carriers are nowadays also regarded as nanomaterials or nanoparticles, and with respect to their specific application also referred to as nanomedicines or nanopharmaceuticals.

The incorporation of anti-infectives into nanoparticulate carrier systems may allow for a high drug loading in some cases, facilitating an increase in effective drug solubility; a masking of undesirable drug effects; a tailoring of anti-infective

pharmacokinetics; or a directly increased permeability. Modifications for example to the particle surface may allow for further improvements, such as a targeted delivery. One of the first examples of a nanoparticulate anti-infective formulation which was granted access to the market is a liposomal formulation of amphotericin B – this formulation remains widely used in clinical settings due to the exhibition of many of the above mentioned advantages (Walsh et al. 1998). Polymeric nanoparticles are also extensively investigated as carriers for anti-infective drugs in several labs around the globe. The protective function of particulate carriers and the possibility for co-loading is also a considerable advantage with respect to delivery of readily-degraded compounds like AMPs. The possibility to incorporate more than one anti-infective compound into particulate carriers, or to combine anti-infective loaded carriers with particles of known antimicrobial substances like gold or silver, constitutes a further advantage to the use of such delivery systems (Huh and Kwon 2011). Significant progress in the development of nanotechnology-based approaches specifically to treat bacterial infections has been made in recent years, leading to the existence of several sophisticated carrier systems. For example, Trojan horse systems made of nanoparticles tagged with folic acid have been shown to mediate an increased activity of the incorporated anti-infective vancomycin in resistant *Staphylococcus aureus* (Chakraborty et al. 2012). The linkage of penicillin G to surface functionalized silica nanoparticles has also shown a restored anti-infective activity even in formerly resistant MRSA (Wang et al. 2014).

Infection-activated delivery systems are another promising approach, being for example composed of chitosan-modified gold nanoparticles which are attached to liposomes or polymeric triple-layered nanogels. Substances like toxins or enzymes which are present in the local environment of a bacterial infection work as a trigger for release of carrier-incorporated anti-infective, allowing for the reduction of potential side effects resulting from systemic anti-infective administration as well as the achievement of high local drug concentrations at the site of infection (Pornpattananangkul et al. 2011; Xiong et al. 2012). Anionic liposomes have also been successfully used to incorporate and deliver plasmid DNA and antisense oligonucleotides into inner bacterial compartments in order to inhibit gene expression in resistant strains (Meng et al. 2009; Fillion et al. 2001). Recently, an SLN-based formulation was successfully used to incorporate and deliver high amounts of a novel quorum sensing inhibitor (QSI), which act as anti-virulence factors by interfering with bacterial cell-cell communication via action on intracellular targets (Miller and Bassler 2001). SLNs with incorporated QSI showed a prolonged release, mucus penetrating ability and an effective delivery to the pulmonary region, as well as an enhanced anti-virulence activity against *Pseudomonas aeruginosa* as compared to the compound alone (Nafee et al. 2014). As these examples illustrate, innovative delivery strategies (along with the search for and optimization of novel anti-infective targets and compounds) offer the potential for overcoming bacterial absorption problems.

3.2.4 Evaluation of Current Status and Future Directions

The combination of EPIs and AMPs with conventional or even new anti-infectives may result in a reduction of undesirable intrinsic anti-infective properties as well as an increased bacterial permeation, leading to higher intracellular drug levels and so an enhanced bacterial bioavailability. Furthermore, carrier systems are able to provide a means of circumventing compound related difficulties, such as unfavorable pharmacokinetics, and to achieve high intracellular drug levels. In this manner such advanced formulation strategies may act to increase the bioavailability of anti-infectives, and for this reason continue to be employed and developed. The treatment of intracellular infections as well as the specific development of permeability enhancing carriers constitutes an important direction of future applications.

4. Conclusion and Outlook

This paper has aimed to give an overview of current difficulties in the treatment of infectious diseases, in particular those caused by Gram negative bacteria. In this respect, the significant bioavailability problems of anti-infective compounds - defined as an inadequate delivery to their (mainly intra-bacterial) sites of action - largely stem from the complex nature of the cell envelope and its formidable barrier function. This barrier function may be even further enhanced by the evolution of resistance mechanisms. Numerous models - *in vitro, in silico* as well as *in cellulo* in nature - may be used in order to increase understanding of permeation processes into or across the envelope, as well as to enable evaluation of how the cell envelope in its entirety or as its individual sub-structures acts as a permeation limiting factor. However, a paucity of quantitative approaches which accurately mimic the overall envelope structure has to be mentioned, meaning that obtained information may lack comprehensiveness. Therefore, new permeation models which more accurately represent the various structural components of the Gram negative bacterial cell envelope, and which are further able to provide quantitative, kinetically- and spatially-resolved permeation data are desperately needed. Such models would also ideally allow for discrimination between active and passive transport processes, and would be applicable as high throughput screening methods in early drug discovery. With respect to anti-infective compounds themselves, the combination of EPIs or AMPs with conventional anti-infectives presents a promising strategy in overcoming bacterial bioavailability problems, enabling the restoration of anti-infective activity even in resistant strains. Particulate delivery systems may similarly facilitate an increase in anti-infective bioavailability, by acting to overcome drawbacks related to the free drug itself; such carrier systems may additionally facilitate a targeted delivery of anti-infectives. Anti-infective formulations which are designed to particularly increase the permeation or

transport of anti-infectives into or across the bacterial cell envelope, or to treat particularly problematic bacteria (such as those which reside within mammalian cells) are still urgently needed however, and would constitute a further significant improvement in anti-infective therapy.

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