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Breaching the Barrier: Quantifying antibiotic permeability across Gram-negative bacterial membranes

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Abstract

The double membrane cell envelope of Gram negative bacteria is a sophisticated barrier that facilitates the uptake of nutrients and protects the organism from toxic compounds. An antibiotic molecule must find its way through the negatively charged lipopolysaccharide layer on the outer surface, pass through either a porin or the hydrophobic layer of the outer membrane, then traverse the hydrophilic peptidoglycan layer only to find another hydrophobic lipid bilayer before it finally enters the cytoplasm, where it typically finds its target. This complex uptake pathway with very different physico-chemical properties is one reason that Gram-negatives are intrinsically protected against multiple classes of antibiotic-like molecules, and is likely the main reason that *in vitro* target based screening programmes have failed to deliver novel antibiotics for these organisms. Due to the lack of general methods available for quantifying the flux of drugs into the cell, little is known about permeation rates, transport pathways and accumulation at the target sites for particular molecules. Here we summarise the current tools available for measuring antibiotic uptake across the different compartments of Gram-negative bacteria.

Keywords: porins, outer membrane proteins, envelope permeability, efflux, antibiotic resistance, label-free, mass spectrometry, fluorescence, electrophysiology

Antimicrobial failure and resistance is now widely recognised across the scientific community as a major threat to the modern medical system[1,2]. A combination of significant drug discovery challenges[3], poor economic incentivisation[1,4] and frankly disastrous misuse in both medicine[5] and particularly in agriculture[6] has led us to the present scenario in which multi-, extreme- and now pan-drug resistant pathogens are leading to patient hospitalisations and mortalities across the globe[7,8]. There is a dire need to refresh the antibiotic pipeline with new drugs, with new modalities of action, in order to address the current lack of treatment options for these infections.

To tackle the Worldwide problem of antibiotic resistance, the European Union currently supports a platform called New Drugs for Bad Bugs (ND4BB, www.ND4BB.eu), which is a broad series of public-private partnerships under the umbrella of the Innovative Medicines Initiative (see IMI, www.imi.europa.eu). A particular bottleneck in the development of new antibiotics is their poor permeability in Gram-negative bacteria, which therefore require high drug doses which in turn lead to toxic side effects[1,9–11]. The IMI sub-project *Translocation* in particular has focussed on the permeability challenge, with a report on their results due to be published soon[9]. Along the same lines, the US-based PEW foundation also recognises low permeability as a major bottleneck, and has a stated goal to “understand and overcome barriers to drug penetration and efflux avoidance for Gram-negative bacteria” (The PEW Charitable Trusts: The PEW Roadmap[12]).

The low drug permeability of Gram-negatives arises due to their complex double membrane cellular envelope[10,13–15], depicted schematically in Figure 1[16]. To reach their (typically) cytoplasmic targets, small molecule antibiotics need to first traverse the outer membrane, which is an asymmetric bilayer containing a lipopolysaccharide (LPS) polymer network on its outer surface. This polymer mesh is a formidable permeability barrier to large molecules

irrespective of whether they are hydrophobic or hydrophilic. On the other hand, smaller solutes like nutrients or antibiotics depend on their ability to translocate through transmembrane β-barrel proteins, known as *porins*[13,17,18], to penetrate the hydrophobic outer membrane. It is also well established that the water-filled porins are typically selective for *hydrophilic* molecules. If the target is cytoplasmic, the therapeutic molecule next has to traverse the cytoplasmic membrane, a phospholipid bilayer that favours the transport of small *hydrophobic* molecules via solubility-diffusion mechanisms[19,20]. It is this dual entry barrier that underpins the ability of Gram-negatives to survive antibiotics and drug-like molecules that might otherwise be active against Gram-positive bacteria. In addition, Gram-negatives also have in-built mechanisms for expelling toxic compounds from their interiors – these so called *efflux pumps* actively recognise and expel antibiotics and other toxins from the cell[21–24]. The net result of this is that pharmaceutical *in vitro* target based screening programmes routinely struggled to deliver an agent active against Gram-negatives, simply because the candidates failed to accumulate in the vicinity of their targets at lethal concentrations. There is thus a very limited subset of molecules capable of breaching Gram-negative permeability barriers and, with the inexorable spreading of resistant strains, we are running out of treatment options at an alarming rate.

Despite the obvious importance of this problem and decades of work, the permeability issues associated with Gram-negative cell envelopes have remained challenging to overcome; we still lack a fundamental understanding of the rules governing molecular transport across these membranes. Early work using radioactive tracer molecules revealed selective uptake across the so-called “outer cell wall” in Gram-negative bacteria and pointed towards the potential role of porins in mediating transport[25,26]. To reduce the number of parameters, porins were isolated and reconstituted into artificial planar lipid membranes[27,28]. Conductance measurements suggested single channel pore sizes of about a nm, a value surprisingly close to that revealed by high resolution X-ray structures a few years later[29]. In a

different approach, outer membrane porins (OMPs) were reconstituted into multilamellar liposomes and kinetic information about transport through the porins was obtained from the osmotic swelling induced by successful solute penetration[30]. These so-called liposome swelling assays became an important tool for understanding the uptake of nutrients or antibiotics through porins. Initial experiments with this approach, which involved measuring the transport of variously sized sugars, established the size exclusion cut-off of the OmpF porin to be about 550 Daltons[31,32]. Ions, amino acids, and small sugars may use general diffusion porins to enter the periplasmic space whereas larger sugars and other molecules need to use dedicated pathways for outer membrane transport[18]. These early studies established the molecular sieving properties of porins, and provided an explanation for the high diffusion rates of these compounds through the outer membrane[33]. Comparison of the diffusion rates of solutes of various sizes gave remarkably reliable values of the channel size with respect to the corresponding crystallographic structure. However, swelling occurs in response to the movement of all the solutes, including components of the buffer, and extreme care is needed when this method is used to study the diffusion of charged solutes[31]. A comparison of some previously available techniques for studying antibiotic permeability was also given by Mortimer and Piddock, including the use of the natural autofluorescence of quinolones to study their accumulation in a range of bacterial species[34].

A number of experimental approaches have thus been utilised to quantify antibiotic permeability in Gram-negatives, spanning both precise *in vitro* techniques for studying individual porins or efflux pumps, as well as whole cell screens. However, what is still missing is a quantitative technique capable of precisely determining drug accumulation in the sub-cellular compartments where their targets are actually located. This is a crucial challenge that the field must overcome; controlled studies on individual porins and efflux pumps provide invaluable information about the precise routes taken by drugs to enter/exit cells, but the extraordinary variety of pathways and proteins in different species make it

experimentally unfeasible to study each and every pathway of interest in reconstituted systems. Furthermore, there is mounting evidence to show that the expression of these proteins is highly dependent on the nutrient microenvironment around the cells[35,36]. Inherent variability in gene expression, especially in efflux pump activity, within an isogenic colony leads to variation in drug uptake and hence in drug tolerance[24,37]. These are all naturally occurring complications which confound attempts at understanding drug uptake at the (sub)cellular level. It is absolutely critical to develop techniques where cells can be studied in their native state, in well controlled microenvironments, rather than after repeated washing and resuspension steps in contrived nutrient conditions.

A number of recent reviews have examined the topic of antibiotic accumulation in Gram-negatives, focussing either on the latest technical developments for quantifying accumulation in cells[38], summarising what we know about the structure of the cell envelope and the challenges associated therein[10], or specifically focussing on efflux systems[39] and porins[40]. However, we believe that an understanding of this complex problem of antibiotic permeation into Gram-negatives will involve insights from both bottom-up biomimetic approaches as well as top-down whole cell measurements, and therefore review here the latest techniques in both general areas. We will discuss recent whole cell assays based on mass spectrometry (MS) and single cell microscopy. However, we will also give an overview of the latest developments in electrophysiology measurements of individual transport channels, as well as recent liposome based transport assays; these techniques combine biomimetic model membranes with microfluidic platforms to study antibiotic transport across lipid bilayers and porins with precise control over the drug mixing and exposure times. We will identify the advantages and drawbacks of current approaches, and suggest how we might combine expertise from different scientific backgrounds to overcome the present technical challenges in the field. Finally, we have also included a brief discussion at the end on the economic challenges facing antibiotic development, which we feel need to be discussed in the

scientific, industrial and regulatory communities in order to ensure that our academic investigations do indeed translate into real-world, clinical impact.

Whole cell accumulation assays with Mass Spectrometry

There have been a number of techniques proposed for measuring antibiotic accumulation via MS. Some directly measure the intracellular concentration of the compound, whereas others measure the change in the extracellular concentration and use this to determine compound accumulation[41,42]. Figure 2 outlines the general workflow of both approaches. Methods measuring the intracellular concentration of the drug are more labour intensive and can involve multiple wash stages such as a silicone oil wash[23,41,43], centrifugation washes[44], and filtration processes[38,45] to remove the extracellular compound. These wash stages potentiate the risk of cell lysis and as such, loss of compound leading to inaccurate results[38]. The silicone oil technique involves layering bacteria that have been incubated with the drug over the oil and pelleting it. The silicone oil is then pipetted off along with the supernatant. The largest intracellular investigations of accumulation utilised silicone oil to limit extracellular binding[41,46]. This is because, despite the increased manual workload, this technique leads to less lysis and a cleaner spectrum due to reduced non-specific binding[46]. The concentration of compounds used in the assays is important; limits of detection restrict the use of highly potent antibacterials which, due to the loss of cellular integrity, leak out of the cells and thus invalidate the assay[23,38].

Richter *et al.* have progressed intracellular assays to screen up to 180 diverse compounds[41]. They sought to discover and define guidelines for compound permeability. It was revealed that in *E. coli*, molecules which are rigid, flat and possess a primary amine group preferentially permeate[41]. This study investigated intracellular concentrations through LC-MS/MS in *E. coli* MG1655[41]. Richter *et al.* tested more compounds than have been

previously tested, but also examined a broader collection of compounds[41]. Another accumulation study by Davis *et al.,* which involved an LC-MS/MS assay on 10 compounds in *E. coli, Bacillus subitilis* and *Mycobacterium smegmatis*, struggled to find distinct rules governing permeability[47]. This may be due to the differences in the cell wall and the cell membranes of these organisms, or even an environmental parameter, such as growth temperature. In addition, the limited diversity of compounds tested also restricted their ability to draw conclusions on permeability.

The potential risk of cell lysis due to various assay preparation steps suggests that deducing the change in the extracellular concentration of the drug due to cellular accumulation might be more useful for high throughput screening[42]. As outlined in Figure 2, the bacteria that have been incubated with the drug are pelleted, following which the supernatant is harvested and analysed[42]. This method requires a minimally permeating cold control to deduce accumulation[38,42]. However, cold controls must also be treated with caution – for instance, Stokes *et al.* showed that cold stress makes *E. coli* susceptible to glycopeptide antibiotics via alterations in the outer membrane[48]. This demonstrates the challenges of performing appropriate controls for accumulation assays in bacteria.

Data generated from these accumulation assays can indicate which compounds accumulate in greater concentrations, and as a result are more permeable. This data, however, does not resolve accumulation in the various subcellular compartments. There has been some progress by Spangler *et al.* involving the use of click-chemistry and MS to better understand subcellular accumulation[49]. This method has unveiled some useful information about the accumulation of a range of drugs which contain an azide group[49]. However, it is not applicable to a large library screening of compounds, as it would require the conjugation of a probe to each compound[49]. An LC-MS/MS study on 132 ligA compounds by Iyer *et al.*

looked at accumulation and on-target activity and reiterated the fact that cytoplasmic accumulation is difficult[46]. Another method used to determine the subcellular localisation of drugs is 3D imaging Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS) by Tian *et al.* Here a cluster ion beam was used to resolve the subcellular localisation of the antibiotics[50]. The C_{60}^{+} method of ionisation utilises a label-free method to reveal the subcellular localisation of ions with a spatial resolution of approximately 300 nm[50]. The equipment used in this work offers exceptional imaging and spectral analysis of compounds. In contrast to standard LC-MS, it is able to combine depth analysis and 2-dimensional imaging, making a 3-dimensional molecular rendering feasible[50]. This technique generates a great deal of data as every pixel in the image contains a spectrum. As a result, the data analysis is complex, as is the experimental setup itself, and it remains to be seen how easily the technique can be transferred into a commercial setting. However, as a label-free technique with sub-micron resolution, it represents an important technological advance that promises to be a valuable addition to the study of compound accumulation in bacterial compartments.

Prochnow *et al.* have also recently reported a new workflow that involves subcellular fractionation via cold osmotic shock, ultrasonic treatment and multiple centrifugation steps to quantify the subcellular accumulation of four different classes of antibiotics in WT and efflux deficient *E. coli* cells[51]. Although the workflow involves multiple steps and hence may not be able to examine antibiotic uptake in real time in various environmental conditions, it is nevertheless another important advance in the field which enables the measurement of a variety of drug classes in a label-free manner directly in the respective vicinities of the drug targets. As an example of the information made available, the authors were able to quantify differences in the concentrations of ciprofloxacin in the periplasm versus the cytoplasm of *E. coli*; correcting for the volume of the respective compartments revealed that the concentration of ciprofloxacin was 6-fold lower in the cytoplasm than in the periplasm. Such

quantitative information will be crucial for the rational design of novel drug candidates in the next stages of antimicrobial drug discovery.

Despite its many advantages, it is important to note that mass spectrometry generates a spectrum which reports the mass to charge ratio of the observed molecule and the intensity of ions at this peak, which is not directly quantitative. The determination of concentrations by ion intensities can be complicated by contaminants and detergents and as such direct quantification of mass spectra is challenging. The current techniques also involve a great deal of manual labour, which is incompatible with high throughput screening (HTS). Preparing the samples for intracellular accumulation not only includes several cycles of centrifugation or silicone oil washing, but also manual lysis methods such as freeze-thaw cycles or sonication. To use MS for HTS, techniques such as acoustic mass spectrometry have been proposed. This involves using an acoustic dispenser coupled to a mass spectrometer[52,53]. A sound wave hits the sample causing the formation of a droplet which is fired into the ionisation beam; this technique can analyse three samples a second[52,53]. Furthermore, the acoustic mass spectrometer is set up in a high throughput 384 well plate format, meaning the amount of compound required is greatly reduced[52,53]. The acoustic coupling bypasses LC-MS loading which increases the speed of analysis, but could mean that complex samples are not separated as efficiently[52].

A single LC-MS run of one compound generates a vast amount of information, but to appropriately deduce accumulation a range of controls are necessary; these include measuring signals from the antibiotic with no bacteria, the bacteria with no antibiotic, a non-permeating control (low temperature, although subject to the caveat about cold controls mentioned above), and a high accumulating control (efflux deficient)[38]. The sheer amount of data generated by these methods suggests the need for machine learning techniques to

analyse the data and predict permeability rules. The work by *Richter et al.* has been amalgamated into a website (http://www.entry-way.org/) where the ability of a proposed molecule to traverse the outer membrane based on predictive rules can be estimated[41]. Thus MS data is now being utilised for "real-world" output, to show how accumulation data can be rationalised into drug design.

Single cell level microscopy

A number of recent techniques, currently published or under development, involve studying antibiotic accumulation at the single-cell level. By combining single cell level uptake information with recently developed mathematical models[54], there is potential for a breakthrough in the field that would enable the prediction of sub-cellular drug localisation using model parameters extracted from the experimental data.

Deep UV autofluorescence microscopy and microfluorimetry has been successfully implemented by the Pagès group and collaborators to study the accumulation of antibiotics in single bacterial cells[55–57]. These techniques typically involve suspending bacterial cells between two quartz coverslips and investigating them with a tunable deep UV light source (a synchrotron)[58]. Bacteria are prepared by culturing them to exponential phase, pelleting them via centrifugation followed by resuspension in a NaPi-$MgCl_2$ buffer; the cells are then mixed with the compounds of interest just before microscopy[58]. Using this technique, the group was able to study the accumulation of fluoroquinolones label-free in *Enterobacteriaceae*, including studies on cells overexpressing the AcrAB efflux system[59]. However, the deep UV fluorescence measurements require a number of controls for separating cellular autofluorescence and crosstalk from the drug signals[58]. Further, deep UV imaging requires quartz optics, including quartz objectives which are not easily available. A number of controls for cell viability must also be performed, since deep UV excitation is detrimental for the cells.

The same group has also worked with fluorescent derivatives of ceftazidime to study intracellular accumulation at the single cell level using microspectrofluorimetry and epifluorimetry[60]. The derivatives accumulated in the periplasm once the outer membrane was permeabilised, and periplasmic accumulation was found to correlate with antibiotic activity[60]. However, the authors also noted that modifications of the compounds with fluorophores changed the antibiotic activity of the drug in *E. coli* strains, which needs to be accounted for when interpreting permeation rates for fluorescent derivatives. The Cooper and Blaskovich groups (Queensland) have also pioneered the development of a range of fluorescent derivatives of antibiotics, which are being applied to single cell level fluorescence assays, including using super resolution structured illumination microscopy (SR-SIM)[61,62]. They have recently reviewed the latest developments in the field, showcasing the various ways that fluorescent antibiotic derivatives are being used to investigate mechanisms of resistance[63].

However, techniques based on single cell fluorescence still, in general, lack the ability to resolve accumulation in subcellular compartments. As mentioned previously in relation to MS approaches, centrifugation and washing steps are less than ideal, and yet without them, it is challenging from single cell microscopy to distinguish drug molecules bound to the outer membrane from drug molecules that actually permeate into the cell. What is also missing is the development of accumulation assays in which the microenvironment and the drug exposure time is well controlled – this is where nanofluidic or microfluidic techniques could provide solutions. Indeed, a nanofluidics assay, combining bacterial trapping in microchannels with controlled drug exposure, was recently reported by the Lee group; they quantified the translocation of clindamycin through wild-type and an efflux deficient *E. coli* strain, although they used an LCMS assay to quantify the clindamycin flux per cell rather than a fluorescence readout[64]. However, although the calculation is performed “per cell”, the

actual measurement accumulates perfusion data across a population of approximately 200 cells. Further, the technique measures the flux of drug molecules across the cell rather than cellular accumulation itself, and it is unclear whether any subcellular concentration information can be inferred from this technique. The technique also relies on having an excellent seal between the cells and the trap channels, which may limit its applicability – different cell types and even isogenic cells in different metabolic states will have different widths and shapes, which would affect the quality of the seals and lead to leakage of the drug molecules between the loading and collection channels. Nevertheless, combining innovative microfabrication and single cell trapping approaches[37] with microfluorimetry or fluorescence has the potential to enable measurements of drug accumulation in well-defined microenvironments. This is important when considering that different growth conditions lead to changes in porin expression[35]; these systems could be used to analyse the role of different media conditions in influencing drug accumulation kinetics. These assays are currently under development, and combining microfluidics with the latest microscopy techniques has immense potential for solving long standing problems in the field.

Raman microscopy and microspectroscopy have also been used to study the uptake and interactions of β-lactamase inhibitors in groups of freeze-dried *E. coli* cells[65,66] in a label-free manner. The Carey group built upon their expertise in detecting different inhibitor-enzyme intermediates with Raman microscopy[67] to study the penetration of clavulanic acid and tazobactam into *E. coli* cells; their inhibitory effects on β-lactamases were also characterised[65]. Although Raman spectroscopy has in the past been technically challenging when used to study bacteria due to low signals and noisy backgrounds, the use of freeze-drying and subsequent difference spectroscopy has now enabled these semi-quantitative measurements in groups of cells[66].

Alternative techniques to measure uptake in cells involve using enzymatic activity inside the bacteria as a readout. For example, the influx of cephalosporins can be measured by coupling their uptake to their hydrolysis by periplasmic β-lactamases[68–70]. However, to connect whole cell experiments with single channel recordings, it is important to be able to quantify the amount of functional protein in the outer membrane. Recently the team of D. Bumann at Biozentrum Basel was able to quantify the number of porins in the outer membrane of *Acinetobacter baumanii* in infected mouse and rat lung tissue[71]. Combining new techniques for single cell level uptake measurements with developments in proteomics would be extremely beneficial for the field, although at present there remain significant technical challenges that still need to be overcome before this can be made a reality.

Latest developments in electrophysiology:

Molecular details of antibiotic transport through specific porin pathways can be obtained from single channel characterisation experiments in “black lipid membranes” or “BLMs”, which are described in detail in Gutsmann *et al.*[72]. Briefly, purified detergent-solubilised channel proteins or proteoliposomes are added to one side of the membrane and spontaneously insert into the bilayer over time. The sequential insertions of open channels in the membrane lead to discrete current jumps due to ion movement through the open channels. The conductance (i.e., the amount of current per unit voltage) of a channel can be obtained by measuring the size of these current jumps. For a number of applications, the reconstitution of proteins into giant unilamellar vesicles and subsequent patch–clamp measurements are advantageous[73]. For this technique, a commercial tool (Port-a-Patch, Nanion Technologies) may be used, which facilitates the experimental handling as the liposome containing preparation is simply pipetted on top of a hole in glass and aspirated to form a giga-seal[74,75]. After insertion, the channel activity can be studied under various conditions (*e.g.* in the presence of DNA, polymers or small molecules). Investigations by the Benz and Rosenbusch groups in the ‘70s and ‘80s established some of the hallmark

properties of the general diffusion porins, such as the relatively large pore size, low ionic selectivity and high opening probability[75–77].

The main advantage of BLM measurements is their ease of access and the low consumption of protein. Although this is a single molecule technique, the information originates from the modulation of the ion current with time; the extracted information is an average over the entire path along the channel. More molecular details about how the molecules move along the channel and their main point of interaction can be obtained in combination with all atom molecular dynamics (MD). Indeed, with the numerous high resolution structures upcoming forthwith, MD simulation is a perfect complementary technique to single channel electrophysiology. For example, some years ago the ion pathways in OmpF were investigated in more detail, and these studies revealed that the paths taken by anions and cations are divergent at the constriction region (CR); cations are drawn close to the negative charges of the L3 loop, and anions flow near the positively charged cluster of the opposite barrel wall (see also Figure 3a)[78–80]. This type of work emphasises the notion that the permeating ions interact with the wall of the channel and that ion movement does not follow simple diffusion. Moreover, measuring the temperature dependent OmpF channel conductance and normalising over the temperature dependent bulk ion conductances revealed a clear deviation from the bulk. An Arrhenius plot allows the quantification of these ion interactions[79,80]. MD modelling revealed two distinct ion pathways when the ion concentration is below 150 mM KCl, whereas above this concentration, ions fill almost the entire pore volume.[79,80]

In a similar manner, substrate permeation is facilitated by an affinity to specific channels. The addition of malto-oligosaccharides to LamB (Maltoporin) from *E. coli* drives the sugar to enter the binding site and block the channel entirely for ions. The occupancy of the binding site with sugar was obtained by measuring the reduction in channel conductance[81]. The permeabilities obtained for malto-oligosaccharides using ion current noise agreed well with

liposome swelling results, except in the case of sucrose[30,81]. Inspection of the structure revealed that sucrose has a binding site but is not able to permeate[82]. It is also possible to extend the noise analysis technique to antibiotic permeation. However the analysis requires a sufficiently strong binding of the drug to the channel, and the antibiotic in the binding site must sufficiently block the ion current[83]. A further point to note is the inherent instability of antibiotics[84]. For example, in the case of ampicillin, a short exposure to basic pH to enhance the solubility can degrade large amounts of the compound.

Binding can be distinguished from transport by applying an external force on charged molecules. As such, an electric field can drag or repel charged permeating molecules, which is then translated into a modification of the residence time in the channel. Thus, measuring the average translocation time for different external electric fields should provide a clear picture[85]. In the case of uncharged molecules, the molecular movement could be modulated by electro-osmotic flow[86]. Most of the porin channels have an excess of one charge species in the constriction zone, thus an externally applied electric field would cause a net unidirectional incoming flow; molecules diffusing with the flow would have shorter residence times (see Figure 3b). In a series of investigations, the specific permeation of α-cyclodextrin across CymA of *Klebsiella oxytoca* was quantified; the individual steps have also been simulated[86]. A more recent suggestion for enhancing the signal for *translocation* is to engineer a barrier for exit within the pore[87]. By comparing the residence times of molecules in the porin in the presence and absence of the barrier on the periplasmic side of the channel, one can identify true permeation events and identify molecules that reach the exit. Starting from the well-studied porin OmpF, a single point mutation at position 181 $OmpF^{E181C}$ was introduced and crosslinked with either Sodium (2-sulfonatoethyl) MethaneThioSulfonate (MTSES) or glutathione. The modification of $OmpF^{E181C}$ by MTSES builds a barrier which is sufficient to block the pathway of norfloxacin through the porin. The modulation of the interaction dwell time allows one to determine the successful permeation of norfloxacin

across wild-type OmpF. This approach might enable the discrimination of blockage events from translocation events for a wide range of substrates while working in the µM range. As the data analysis is straightforward, parallelisation of the experiments might be possible. A potential application of this technique could include screening for molecular structures to improve the permeability of antibiotics.

A different approach involves the use of an unbalanced charge accumulation[84,88]. Creating a concentration gradient between both sides of the channel induces a concentration driven flux (see Figure 3c). In most cases, one of the ions diffuses slower than the counter ion; the difference in flux creates a *diffusion potential*. Measuring the diffusion potential as a function of the concentration gradient reveals relative fluxes. In combination with a single channel conductance measurement, a more quantitative analysis is possible. Note that the cation's permeation strongly depends on that of the anion and vice-versa and thus the permeability is not a simple constant but varies with concentration and the counterions. In the case of charged antibiotic molecules, their channel permeability can be estimated by the application of a concentration gradient and the measurement of the potential built up by an unequal permeation of the cation versus the anion. This technique is similar to a selectivity measurement, and a numerical solution based on the Goldman-Hodgkin-Katz ion current equation can be used. The permeation of three β-lactamase inhibitors (avibactam, sulbactam and tazobactam) through OmpF and OmpC orthologues from four enterobacterial species was recently characterised using this approach. The information from electrophysiology can be combined with existing high resolution structures and modelling to obtain the energy barrier. For example, in the case of OmpF, the energy barrier for small ions is low and broad whereas for the beta-lactam inhibitors the barrier is substantially higher and narrower (see Figure 3d). Surprisingly there is also a shallow affinity site just before the entry at the extracellular side[88]. Well-designed single channel conductance measurements, in combination with MD simulations, thus continue to be a

powerful tool for investigating transport through individual components of the bacterial cell envelope at the molecular level.

Biomimetic liposome assays:

Liposomes or lipid vesicles are versatile tools for studying membrane processes[89]. We have already discussed how multilamellar (multiple lipid bilayers) liposomes were used in swelling assays to study solute transport through porins. On the other hand, unilamellar vesicles containing a single lipid bilayer can be used to mimic the lipid bilayer of cell membranes, with precise control of the lipid content and composition of the membrane. Small unilamellar vesicles (SUVs) typically have diameters between 50 – 200 nm, large unilamellar vesicles (LUVs) range from 200 – 1000 nm in diameter, whereas giant unilamellar vesicles (GUVs) typically have diameters ranging from 1 – 100 μm. SUVs and LUVs can be formed relatively easily and quickly using lipid extrusion techniques[90], and are then often subjected to bulk assays such as spectrofluorimetry where transport or binding processes are studied using changes in the fluorescence or absorbance of the bulk solution as a whole over time[91]. This is typically correlated with the leakage of a dye or fluorophore encapsulated within the vesicles, or alternatively, the transport of a solute across the SUV bilayers which then reacts with an encapsulated target to change the fluorescence of the solution[92]. The latter principle was combined with TIRF microscopy and microfluidics by the Dittrich group to study tetracycline transport across SUV membranes, where the SUVs were immobilized on a glass substrate via an avidin-biotin bond[93]. The process involved encapsulating an Eu^{3+} salt inside the vesicles, which formed a fluorescent complex with tetracycline as it diffused across the SUV bilayers[93]. They used the technique to investigate the influence of membrane composition on permeation rates, and showed that although drug lipophilicity as predicted by traditional octanol-water partition coefficients correlates well with membrane permeability, it is not the only significant parameter[93].

Similar results were observed with a related approach, developed in the Keyser laboratory at the University of Cambridge, where GUVs were used as the model membranes. The advantage of using GUVs is that membrane transport can be investigated at the single vesicle level using standard microscopy approaches[94]. However, traditional techniques using GUVs are limited in throughput – the GUVs are typically suspended in a droplet on a coverslip, and when fluorescent compounds are added, their uptake is measured by studying the change in fluorescence inside the GUVs. However, this is limited in throughput to the number of vesicles in the field of view of the microscope[95]. The other problem with such experiments is that the initial time of drug arrival and its concentration in the vicinity of the GUVs are both ill defined.

These technical problems were overcome using microfluidics. The GUVs were formed off chip using electroformation[96], and were then introduced into one of the inlets of a simple T-junction microfluidic device, whose channels were approximately 40 μm in width and height[97,98]. The drug of interest was added using the second inlet of the T-junction, and the flows were controlled by applying suction at the outlet using a syringe pump[97]. The design enables the equal mixing of the vesicle fluid stream with the antibiotic at the T junction, and thus the time of vesicle exposure to the drug as well as the drug concentration surrounding the vesicle are both well defined[97]. The use of a flow system enabled measurements on hundreds of vesicles rather than the tens of GUVs normally studied in such assays[94,95]. Importantly, the technique utilised the autofluorescence of quinolone antibiotics in the ultraviolet (λ_{ex} = 340 nm) to track their uptake in GUVs label-free; this simplifies the analysis since there are no secondary binding interactions that need to be accounted for when calculating the accumulation of the drug in the GUVs. Furthermore, unlike the industry standard PAMPA (parallel artificial membrane permeability assay) technique, there are no

significant complications from unstirred water layers[98], which may affect the interpretation of compound permeability[99].

Using this technique, the group also confirmed that lipid composition plays a critical role in antibiotic permeability[100]. For example, at pH 7, norfloxacin permeability was found to be an order of magnitude higher for GUVs made of DOPC lipids as compared to DPhPC – these lipids have the same phosphatidylcholine (PC) head-groups but differ in the composition of their hydrocarbon tails, with the branched methyl groups in the DPhPC tail believed to be responsible for its lower permeability to small molecules[100]. The group investigated norfloxacin transport across GUV membranes formed from a range of different lipid mixtures, and further reiterated the need to quantify drug transport across the specific lipid bilayer composition of interest[100].

Such investigations will also be particularly important for comparing the permeation of drugs across Gram-positive membranes and the cytoplasmic membrane of Gram-negatives. There is some debate in the field about the relevance of the cytoplasmic membrane of Gram-negatives as a barrier, since a wide range of drugs are able to accumulate in the cytoplasm of, for instance, *E. coli* cells with compromised outer membranes[101]. However, as mentioned in the introduction, the difficulty with Gram-negative cytoplasmic entry arises from the apparently orthogonal sieving properties of the outer versus the cytoplasmic membrane, as has been extensively discussed previously[10,101]. It is also worth noting that Gram-positive membranes and the cytoplasmic membranes of Gram-negatives differ significantly in their lipid compositions[102]. This is likely to lead to quantifiable differences in drug transport rates across these membranes, which should be a future avenue for liposome based investigations.

Vesicles are thus good models for studying passive transport across the cytoplasmic membrane of bacteria, where solubility and diffusion across the lipid bilayer are important. However, transport across the outer membrane is generally governed by the porins. We have reviewed electrophysiology approaches to study antibiotic binding and transport through individual porins in a separate section. However, although these measurements have proved invaluable in understanding the molecular interactions between different drugs and channels, they are limited by the fact that actual fluxes are still challenging to measure.

To solve this conundrum, the authors (JC and MW) collaborated to reconstitute OmpF into GUV membranes, creating proteoliposomes which could then be studied in the microfluidic transport assay described above (Figure 4)[103]. This provides a controlled, bottom-up approach to studying antibiotic transport across biomimetic bacterial membranes – norfloxacin transport was first quantified across pure GUV lipid membranes, and then the same experiments were performed with the OmpF containing proteoliposomes. The experiments directly measured the enhancement in flux due to the porins in the membrane, paving the way for quantifying antibiotic permeability through transport proteins of interest using a direct optical readout[103].

A difficulty with GUV based approaches has been that traditional vesicle formation protocols have a number of limitations. Electroformation, for example, involves applying alternating electric fields to bud lipid films off a conducting surface – this makes the use of physiological salt conditions challenging[104]. The populations also contain vesicles of varying diameters, and there are often difficulties associated with batch-to-batch variability, especially in the presence of salts. Furthermore, there are various reconstitution techniques for incorporating proteins in GUVs, but they each have certain drawbacks and there is no one standard technique that works better than the rest[105]. In order to overcome these challenges, a

number of groups have now developed microfluidic GUV formation protocols[106]. One promising technique is octanol-assisted liposome assembly (OLA), developed by the Dekker group in Delft[107]. This involves a microfluidic jetting technique where lipids are dissolved in octanol as the organic phase – post GUV formation, the octanol forms a pocket which then spontaneously buds off as a droplet. The octanol can be separated[108] and then the pure GUVs analysed downstream of formation. We have now successfully integrated the OLA platform with an antibiotic transport assay downstream of GUV formation, thus providing a complete lab-on-chip platform to create and test vesicles in physiological salt conditions[109].

These new microfluidic liposome formation platforms will also enable the investigation of an important question with regards to the transport of charged molecules across cytoplasmic membranes. As mentioned previously, the challenge with Gram-negative cytoplasmic uptake is that charged, hydrophilic molecules preferentially permeate the outer membrane, but then face a hydrophobic cytoplasmic lipid membrane across which their transport rates are much slower than that of neutral uncharged species; this has been quantified directly for drugs in different charge states[98]. It has been proposed that the transport of charged molecules across the cytoplasmic membrane may be mediated by the proton motive force and electrochemical gradients, leading to energy-dependent but carrier- independent mechanisms of transport[10]. In the past, membrane vesicles from species such as *Lactococcus lactis* and *Leuconostoc mesenteroides* have been coupled with membrane potentials to examine the transport of amino acids, citrate and malate[110,111], but detailed investigations on antibiotic uptake in such conditions have not yet been realised. Using the OLA formation platform, we have recently demonstrated that it is possible to trap thousands of vesicles encapsulating a pH sensitive dye in physical traps[112]; the vesicles are then subjected to antibiotic treatment over a time period of hours. This platform could in the future be utilised to study the role of electrochemical and pH gradients on drug transport by utilising the high encapsulation efficiency of the OLA platform. One could potentially set up precise

electrochemical and pH gradients between the interior and the exterior of these synthetic vesicles and then flush charged antibiotics into the system, monitoring the uptake of the drugs via a fluorescence based readout similar to that described above. Such investigations could be used to quantify the role of electrochemical potentials on the uptake of charged antibiotics across model cytoplasmic membranes.

Vesicles are therefore promising model systems for the study of passive antibiotic transport across specific lipid and porin pathways in bacteria. However, they have also been used to study active efflux via the reconstitution of bacterial efflux systems in proteoliposomes[113]. The LmrP multidrug transporter from *Lactococcus lactis* was successfully incorporated in a proteoliposome system by the van Veen group in Cambridge, where it was used to study Calcium transport[113]. Complexes of OprM and MexA, two proteins from the MexA-MexB-OprM multidrug efflux pump of *Pseudomonas aeruginosa* have also been reconstituted in SUVs and LUVs for the cryo-electron tomography of the protein complexes, suggesting that entire MDR efflux pump complexes can be successfully reconstituted[114]. The Picard group combined proteoliposomes containing the MexAB and the OprM parts of the complex to develop an assay that measured energy-dependent substrate transport in a system designed to mimic the Gram-negative double membrane[115]. The authors proposed that their model system be used to screen for inhibitors of the efflux pump, which would be valuable assets for restoring the activity of older antibiotics that would otherwise be subjected to efflux from the cell[115].

Protein reconstitution in SUVs or in multilamellar vesicles has traditionally been far easier than reconstitution in GUVs. Therefore, techniques to form GUV sized proteoliposomes from SUV proteoliposomes are of great interest, enabling the screening of multiple different bacterial transport proteins using the modern microfluidics approaches described above. The

Spatz group has recently published a technique[116] that uses SUVs encapsulated in block-copolymer surfactant-stabilized water-in-oil droplets; by tuning the charge at the inner interface of the droplet, SUVs can be made to adsorb at the interface forming droplet-stabilized (ds) GUVs. They demonstrated a simple step to release these GUVs from the surfactant and oil, resulting in a high yield of GUV formation[116]. This paves the way for converting SUV-proteoliposomes into GUV-proteoliposomes which could then be investigated as described above, with high resolution microscopy/microfluidics approaches.

Both OLA and the dsGUV techniques are relatively recent developments in the field, and hold great promise for the construction of truly biomimetic models of bacterial membranes. The next technical leap would involve the *controlled* formation of double bilayers to better mimic Gram-negative cell envelopes. If this were to be achieved, it is not inconceivable to imagine the reconstitution of entire transporter complexes such as MDR efflux pumps, spanning the “inner” bilayer, the “periplasmic” inter-membrane space and the “outer” bilayer, in these model systems. By subjecting them to the antibiotic transport measurements described above, a wealth of information could be untapped regarding the fluxes, activation barriers and selectivity of these crucial antibiotic transport pathways in bacteria.

A note on the economics of antibiotic research and development:

We end this review with a slight diversion from our topic of studying transport across bacterial membranes. As professional scientists, it is sometimes easy to lose track of the bigger picture when working on the technical minutiae of a problem. Why are we interested in understanding the details of antibiotic transport? Obviously, the major aim is to exploit our knowledge and techniques to develop more effective antibiotics and to circumvent existing drug resistance and tolerance mechanisms. However, to translate this work into antibiotic development and ultimately, to the benefit of actual patients, we require a drug development

ecosystem whereby promising academic discoveries are implemented in a timely and effective manner in biotechnology and pharmaceutical companies.

However, as the field knows very well, the antibiotic pipeline has dried up – no new broad spectrum antibiotic classes have been brought to the market in the past 5 decades[117]. This is not only a scientific but also an economic problem, as has been recognised by numerous studies on the issue[1,2,118,119]. In a volume-based payment system, revenues are driven by sales, sales are driven by prescriptions; but with antibiotics, the more prescriptions there are, the faster resistance develops, thereby lowering the intrinsic value of the drug[120]. New antibiotics typically need to be preserved for last-resort cases in which all other options have been exhausted, which also translates to lower sales. Even extremely promising drug candidates such as Novartis' new monobactam derivative LYS228 are currently being pulled from the pipeline, with the company seeking to outlicense the therapeutic[121]. Unless the economic challenges are resolved, irrespective of the scientific advances made in understanding the basic biology and pharmacokinetics, real world impact in clinics will not be observed.

In his seminal review of the field, Lord O'Neill stressed the importance of tackling this antibiotic development and supply problem[1]. Both he and others[120,121] have suggested giving market entry rewards of the order of $1 Billion to the developers of new antibiotics, to both develop and actually manufacture stocks of new antibiotics which would be made available in clinical settings as appropriate. It is pertinent here to stress the importance of manufacturing and *maintaining* stock supplies, even if the drugs are used sparingly; it is worrying to note that economic considerations are leading to supply failures of commonly prescribed antibiotics in today's market, further exacerbating problems in infectious disease control[122]. Market entry rewards need participation from various governments, to tackle what

is very much a global problem – pathogens do not recognise passport controls. A global antibiotics reward fund, sponsored by the World's major economies, would be able to address this issue. Along these lines, it is heartening to note that the UK government has, in January 2019, announced a 20-year vision and a 5-year national action plan on antimicrobial resistance, which includes proposals to address the antibiotic market failure crisis and incentivise antibiotic development[123].

Besides governments, it is also worth considering the massive public and charitable engagement that other fields of medical research have generated, and contrast that with the case of antibiotic research. In 2017-18, Cancer Research UK raised £634 Million[124]. The British Heart Foundation raised £159 Million in 2016-17[125]. In contrast, Antibiotics Research UK, the only charity in the World focusing specifically on antimicrobial resistance, had an income of £235,000 in 2016-17[126], *three orders of magnitude lower* than the others. These figures show just how much scope there is to increase funding for this crucial area of research through public engagement. Public awareness and charity involvement would boost basic research into antibiotics, which would help refresh the development pipeline and potentially lower entry barriers for pharmaceutical companies looking to enter or re-enter the field. We urge the scientific community, policy makers, heads of charitable organisations and the general public across the globe to consider these issues seriously and begin planning ways to massively increase funding to this vital sector, both in the areas of basic research such as the transport studies discussed here, as well as in the translational programmes involved in getting new drugs from the bench into the clinic.

Concluding remarks:

Despite the challenging economic environment for antibiotic R&D in general, research into drug permeation in bacteria has developed considerably over the past decade. These new

techniques will pave the way for identifying and characterising the physicochemical properties of permeating compounds, which should help guide medicinal chemists in their quest for discovering novel antimicrobials with Gram-negative activity. The various methodological innovations involving mass spectrometry, fluorimetry and microfluidic assays also offer new opportunities to better understand the relationships between genetic control of the transport pathways and bacterial adaptation to diverse environmental stresses. Real-time measurements of the intrabacterial accumulation of specific molecules, inducers, inhibitors and antimicrobial drugs represent important breakthroughs which have been made possible by collaborations extending across traditional scientific boundaries. Recent developments in whole cell assays, using MS and single cell microscopy, have been vital to the field and show great promise in providing crucial information for the rational design of new antibiotics. Complementary research programmes with continuous feedback between the whole cell and model system based transport assays will be crucial for generating a holistic understanding of transport in these systems. Such programmes will require molecular biologists, microbiologists, geneticists, medicinal chemists, biophysicists, mathematical modellers, bio-engineers and image analysis experts to succeed. Although the problem is extremely complex, the recent developments reviewed here suggest that, with the right scientific networks and funding modalities, a more fundamental understanding of drug permeation in Gram-negatives is accessible. This will be invaluable in the search for new drugs to tackle the next generation of resistant pathogens.

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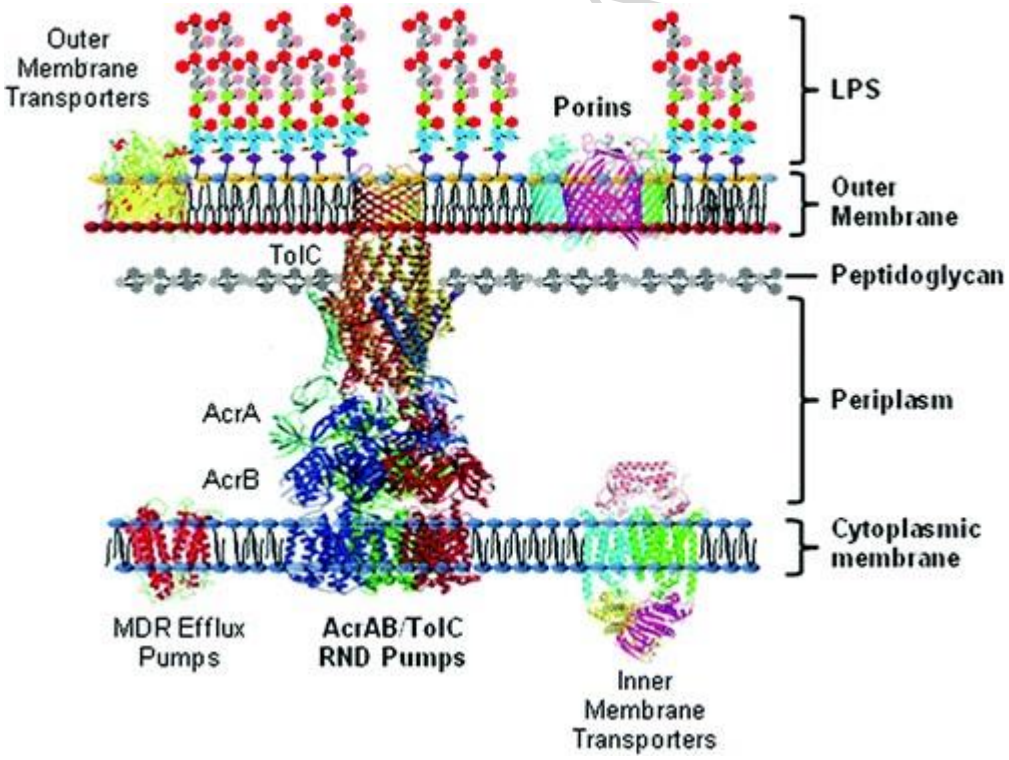


Figure 1. Schematic of the Gram-negative cell envelope. Adapted with permission from Manchester, J. I., Buurman, E. T., Bisacchi, G. S. and McLaughlin, R. E. *"Molecular determinants of AcrB-mediated bacterial efflux implications for drug discovery." J. Med. Chem.* **55,** 2532–2537 (2012). Copyright (2012) American Chemical Society.

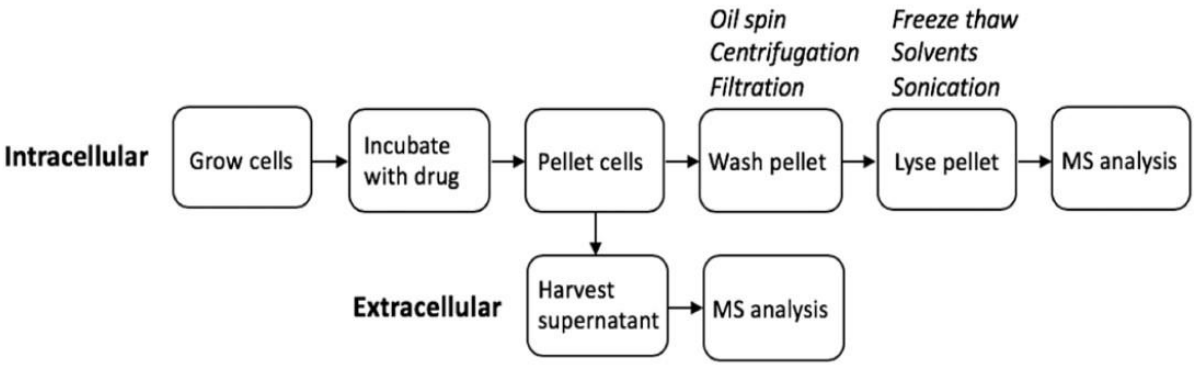


Figure 2. General workflow of Mass Spectrometry assays. An outline of the general workflow utilised in mass spectrometry based intracellular and extracellular drug concentration measurements.

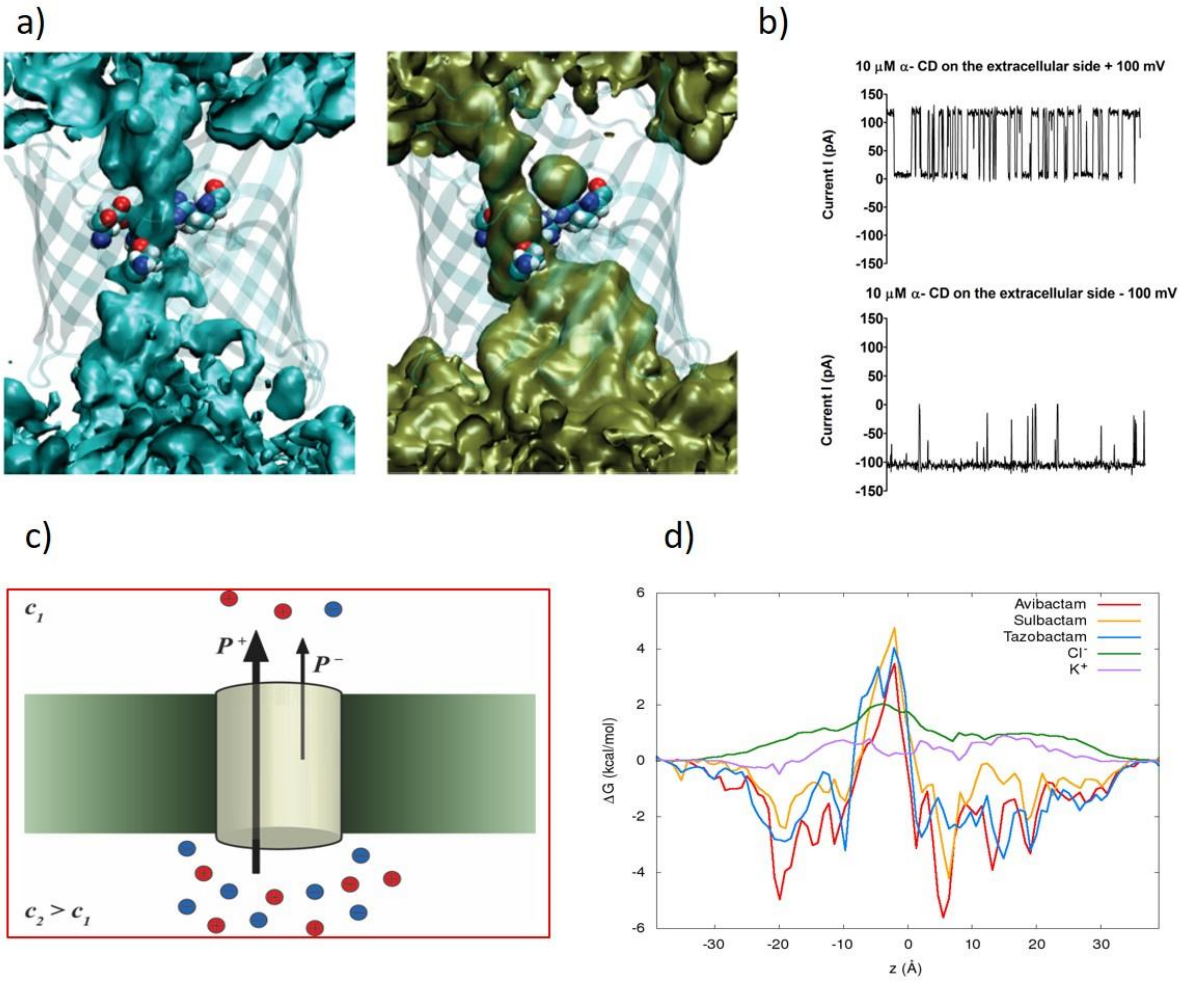


Figure 3(a). Iso-density surfaces of the Cl^- (left) and K^+ ion densities (right) in an OmpF channel averaged over the full trajectory. The important residues in the constriction zone are shown as well. For details, please see Pezeshki *et al.* (2009)[80]. Reprinted from *Biophysical Journal* Vol. 97, Soroosh Pezeshki, Catalin Chimerel, Andrey N. Bessonov, Mathias Winterhalter and Ulrich Kleinekathöfer, "*Understanding Ion Conductance on a Molecular Level: An All-Atom Modeling of the Bacterial Porin OmpF*", pages 1898-1906. Copyright (2009), with permission from Elsevier.

(b). Effect of electroosmotic flow. Typical ion-current recordings at positive voltage +100 mV (top) and negative voltage −100 mV (bottom) in the presence of 10 μM α-CD on the extracellular side of CymA in 1 M KCl. Here, the electroosmotic flow drags the neutral α-CD into the channel and briefly blocks the ion current through the channel. Reversing the voltage reverses the flow direction and only a few α-CD molecules reach the channel, with substantially shorter residence times. Variation of the applied external transmembrane voltage will modulate the residence times and allows one to determine whether or not translocation was successful[86]. Reprinted from *Biophysical Journal* Vol. 110, Satya Prathyusha Bhamidimarri, Jigneshkumar Dahyabhai Prajapati, Bert van den Berg, Mathias Winterhalter and Ulrich Kleinekathöfer, "*Role of Electroosmosis in the Permeation of Neutral Molecules: CymA and Cyclodextrin as an Example*", pages 600-611. Copyright (2016), with permission from Elsevier.

(c). For charged molecules, a concentration gradient can be applied driving cations and anions through the channel. A difference in the electrophoretic mobility causes a diffusion potential and allows one to identify translocation, and to distinguish translocation

events from binding. Combining this with single channel recordings allows one to estimate the flux.

(d). All atom modelling performed in the group of M. Ceccarelli shows the energy barrier for translocation. OmpF as a cation selective channel has a low and broad barrier for K^+ and a higher one for Cl^-. In contrast, beta-lactam inhibitors like Avibactam, Sulbactam or Tazobactam have sharp energy barriers[88]. Reprinted in part with permission from Ghai, I., Pira, A., Scorciapino M. A., Bodrenko, I., Benier, L., Ceccarelli, M., Winterhalter, M. and Wagner, R., "*General Method to Determine the Flux of Charged Molecules through Nanopores Applied to β-Lactamase Inhibitors and OmpF*". *J. Phys. Chem. Lett.* **8,** 1295–1301 (2017). Copyright (2017) American Chemical Society.

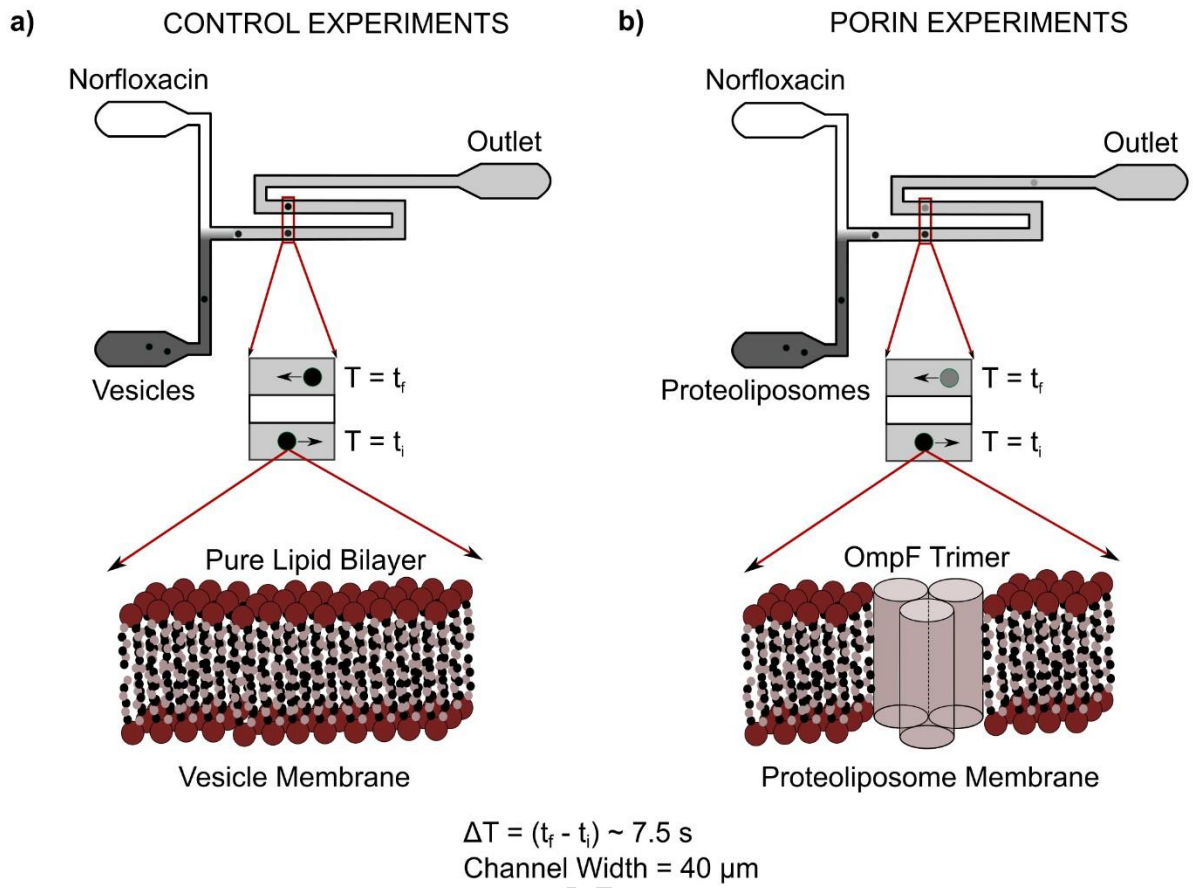
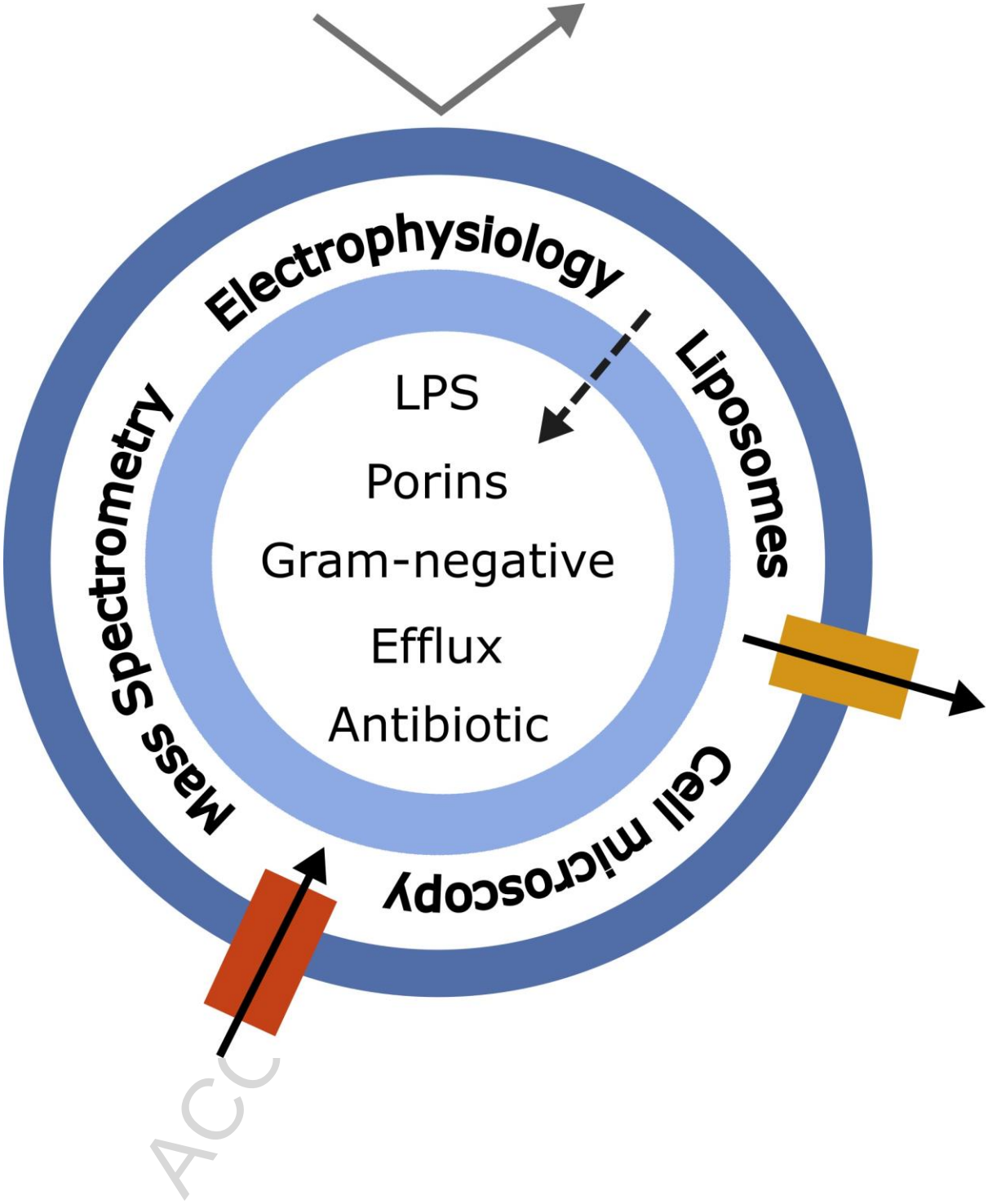


Figure 4. Schematic of proteoliposome based microfluidic assays for studying antibiotic transport across biomimetic membranes. Liposomes and OmpF embedded proteoliposome model membranes are mixed with an autofluorescent antibiotic (norfloxacin, λ_{ex} = 340 nm) in a T-junction microfluidic device. As the control liposomes (a) or proteoliposomes (b) move through the bath of the drug, they accumulate the drug at different rates. Video fluorescence microscopy at multiple time points is used to determine the permeability coefficient of the drug crossing the model membranes. The difference in the permeabilities of the liposomes and proteoliposomes to the drug is used to quantify antibiotic flux through the porins in the proteoliposome membranes. The number of porins per proteoliposome is quantified separately using electrophysiology. Figure reprinted with permission from Cama, J., Bajaj, H., Pagliara, S., Maier, T., Braun, Y., Winterhalter, M. and Keyser, U. F. "*Quantification of Fluoroquinolone Uptake through the Outer Membrane Channel OmpF of Escherichia coli*." *J. Am. Chem. Soc.* **137,** 13836–13843 (2015). Copyright (2015) American Chemical Society.



Breaching the Barrier: Quantifying antibiotic permeability across Gram-negative bacterial membranes

Jehangir Cama*, Abby Mae Henney and Mathias Winterhalter*

Highlights

- Review of techniques to study antibiotic permeation in Gram-negative bacteria
- Latest developments in mass spectrometry and single cell level drug uptake studies
- Innovations in model membrane assays including electrophysiology and microfluidics