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The bacterial cell envelope as delimiter of anti-infective bioavailability – An *in vitro* permeation model of the Gram-negative bacterial inner membrane

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ABSTRACT

Gram-negative bacteria possess a unique and complex cell envelope, composed of an inner and outer membrane separated by an intermediate cell wall-containing periplasm. This tripartite structure acts intrinsically as a significant biological barrier, often limiting the permeation of anti-infectives, and so preventing such drugs from reaching their target. Furthermore, identification of the specific permeation-limiting envelope component proves difficult in the case of many anti-infectives, due to the challenges associated with isolation of individual cell envelope structures in bacterial culture. The development of an *in vitro* permeation model of the Gram-negative inner membrane, prepared by repeated coating of physiologically-relevant phospholipids on Transwell® filter inserts, is therefore reported, as a first step in the development of an overall cell envelope model. Characterization and permeability investigations of model compounds as well as anti-infectives confirmed the suitability of the model for quantitative and kinetically-resolved permeability assessment, and additionally confirmed the importance of employing bacteria-specific base materials for more accurate mimicking of the inner membrane lipid composition - both advantages compared to the majority of existing *in vitro* approaches. Additional incorporation of further elements of the Gram-negative bacterial cell envelope could ultimately facilitate model application as a screening tool in anti-infective drug discovery or formulation development.

1. Introduction

The increasingly reported occurrence of multidrug-resistant bacteria, particularly those of the Gram-negative classification, constitutes a growing threat to the state of health worldwide [1,2]. The up-regulation and evolution of bacterial resistance mechanisms, leading to inadequate drug levels at target sites, in fact acts to exacerbate the already challenging task of successfully delivering anti-infective compounds or formulations into or across the cell envelope [3]. The unique and complex structure of the Gram-negative bacterial envelope operates intrinsically as a significant barrier, preventing the attainment of sufficient drug levels at required sites of action in many instances [4,5]. The envelope itself consists of an inner membrane (IM) of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin as principal phospholipid (PL) components, together with an asymmetric outer membrane (OM) composed of a PL-containing inner leaflet and a lipopolysaccharide-containing outer leaflet. These two membrane structures, additionally incorporating aspects of active transport, are chiefly responsible for the intrinsic barrier properties of the envelope, which is therefore

Abbreviations: CL, cardiolipin; ER, electrical resistance; IM, inner membrane; KRB, Krebs-Ringer buffer; LC, liquid condensed; LE, liquid expanded; MIC, minimal inhibitory concentration; OM, outer membrane; PC, phosphatidylcholine; PL, phospholipid; P_{app}, apparent permeability coefficient; PBS, phosphate buffered saline; PMB, polymyxin B; POPE, 1-hexadecanoyl-2-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine; POPG, 1-hexadecanoyl-2-(9*Z*-octadecenoyl)-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); PVPA, phospholipid vesicle-based permeation assay; SE, standard error of the mean; SEM, scanning electron microscopy; UHPLC, ultra-high performance liquid chromatography.

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commonly termed as a two-membrane barrier [6]. In addition however, the periplasmic space separating the IM and OM serves as an area of high metabolic activity [7], and also houses the peptidoglycan cell wall (a much thinner structure in comparison to Gram-positive bacteria). The described intrinsic and resistance-compounded difficulties in achieving adequate drug levels at bacterial target sites, together with the present low flow within the antibiotic development pipeline, both contribute to a common inability to successfully treat Gram-negative bacterial infections. Such difficulties can ultimately and collectively be regarded as symptoms of a bacterial bioavailability problem [8], which is of vital importance to address. The development of new anti-infective compound classes, the discovery of new targets, and the advent of novel delivery strategies which facilitate effective anti-infective drug penetration into or completely across the cell envelope (in order to reach intracellular sites of action) therefore all constitute important areas of research in this respect. In addition, research efforts within these areas require an increased understanding of and ability to investigate bacterial permeation processes - a difficult task to achieve currently '*in cellulo*' due to numerous associated challenges [8]. As such, a further research need to be addressed is the requirement for models which allow for the characterization and quantification of anti-infective permeation across the Gram-negative bacterial cell envelope. Such models would provide complementary information to that obtained from established, *in cellulo* efficacy testing approaches (such as determination of minimum inhibitory concentrations (MIC)), allowing for optimization of drug candidates with respect to their target interaction as well as their ability to sufficiently permeate the envelope barrier [9].

A variety of *in vitro* models in fact already exist for investigating interactions between anti-infective compounds and bacterial cell envelope components; these can generally be classified as electrophysiology models [10,11], Langmuir films [12] and vesicle-based assays [13]. While all such models are able to provide insight into bacterial permeation processes, they also demonstrate several shortcomings. For instance, most focus on approximating the IM or the OM alone, rather than both structures together (although some progress in this respect has been recently made [14]). Furthermore, the PL composition of existing IM models often deviates from that found in Gram-negative bacteria, in terms of either character or ratio [15]. The majority of the available approaches also do not allow for the quantification of permeation processes [14,16–18], an important ability which would allow for more in-depth and accurate characterization of the way in which anti-infective compounds and formulations interact with the bacterial envelope barrier [19]. Hence, there is a great need for new models which represent the entire Gram-negative bacterial envelope with respect to both composition and structure, and which are specifically designed to yield high content, quantitative permeation information in a kinetically- and ultimately spatially-resolved manner.

As a first step in the production of an overall envelope model, this work is aimed at designing and characterizing an *in vitro* model of the Gram-negative bacterial IM employing bacteria-specific PLs, which is explicitly designed to quantify the passive permeation kinetics of anti-infectives. A Transwell®-based setup, mimicking the conventional procedure to assess permeation through mammalian cell barriers, was employed for the model preparation process. An existing approach for production of lipid-based mammalian membrane models – the phospholipid vesicle-based permeation assay (PVPA) [20] - was adapted in order to prepare the bacterial IM model, utilizing a bacteria-specific lipid composition as found in the IM of Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* [21]. The resulting preparation procedure was also employed to produce a model consisting solely of phosphatidylcholine, as a major phospholipid in mammalian cell membranes [20]. The IM model and the phosphatidylcholine-containing mammalian model ('mammalian comparator') were then directly compared at each stage of IM characterization and in subsequent permeability studies. This comparison was made in order to discern any lipid-dependent differences between the models in terms of structure and function, and in doing so, to clearly demonstrate the need to adapt an already existing mammalian lipid-based model using bacteria-relevant materials. Models were characterized with respect to the interfacial behavior of their component lipids, as well as integrity and robustness, topography, and thickness. Furthermore, sets of model compounds including anti-infectives were utilized to ultimately assess the impact of model lipid composition on permeability behavior, and to highlight the ability to obtain quantitative and kinetically-resolved permeation data.

2. Material and methods

2.1. Materials

1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (POPE), 1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) (POPG) and 1,1′,2,2′-tetra-(9Z-octadecenoyl) cardiolipin (sodium salt) (CL) purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) were used for the IM model preparation. Egg phosphatidylcholine (PC, Lipoid E80) was kindly donated by Lipoid GmbH (Ludwigshafen, Germany), and employed for the mammalian comparator model. Polycarbonate filters with a pore size of 800 nm (Merck Millipore, Darmstadt, Germany) were used for liposome extrusion. Commercially available cell culture inserts (Transwell® permeable supports 3460) were obtained from Corning Inc. (Acton, MA, USA). Calcein, sodium fluorescein, rhodamine 123, rhodamine B, rhodamine B isothiocyanate, atenolol, metoprolol tartrate, timolol maleate, nadolol, acebutolol hydrochloride and alprenolol hydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA) served as model drugs. Polymyxin B (PMB), minocycline hydrochloride and ciprofloxacin hydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA) were employed as anti-infective agents. All reagents for ultra-high performance liquid chromatography (UHPLC) were purchased from VWR (Radnor, PA, USA). All other chemicals and solvents were of at least analytical grade.

2.2. Methods

2.2.1. Langmuir trough experiments

Surface pressure-area π-A measurements of lipid monolayers composed of pure bacteria-relevant PLs (POPE, POPG, CL), their 70:20:10 weight mixture [21], or pure PC were performed using a thermostated Langmuir film trough (775.75 cm^2, Biolin Scientific, Finland) enclosed in a plexiglas box. Experiments were performed at 294 and 303 K (21 ± 1 °C and 30 ± 1 °C respectively). PLs were dissolved in a mixture of chloroform and methanol (9:1 v/v) to form solutions of 1×10^{15} molecules/μl. These solutions were then used to spread PLs at the air/buffer interface, following subphase cleaning by suction. After PL deposition, the system was left for 15 min to allow complete evaporation of the organic solvents. Monolayer compression was then performed at a speed of 5 $Å^2 * molecule^{-1} * min^{-1}$. The results reported are mean values of at least two measurements. The surface compressional moduli (K) of monolayers were calculated from Eq. (1):

$$K = -A * \left(\frac{d\pi}{dA}\right) \quad (1)$$

where A is the PL molecular area ($Å^2$), dπ the surface pressure change (mN/m) and dA is the change in the molecular area.

2.2.2. Model preparation

Both the bacterial IM and the mammalian comparator model were produced by adapting the PVPA approach [20]. Liposomes composed of bacteria- or mammal-relevant PLs were first prepared *via* the lipid film hydration method [22]. Briefly, POPE, POPG and CL were used in a 70:20:10 weight ratio, as a bacteria-specific PL mixture. PC was used