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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.**

# Supplementary Material

**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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**Table S1** . Key physicochemical parameters and characteristics of fluorescent dyes used for permeability investigations, as well as parameters utilized for compound quantification.

| Fluorescent dye | $logD_{(pH\ 7.4)}$ [a)] | $M_w$ (g/ mol) | PSA ($A^2$) [b)] | H-bond donors/ acceptors [b)] | Initial donor concentration (µM) | $\lambda_{exc}$ (nm) – $\lambda_{em}$ (nm) |
|---|---|---|---|---|---|---|
| Calcein | -1.46 | 622.5 | 232 | 6 / 5 | 22 | 470 - 530 |
| Fluorescein | -0.43 | 376.2 | 76 | 2 / 5 | 26.6 | 485 - 530 |
| Rhodamine 123 | 1.17 | 344.4 | 85.4 | 3 / 5 | 14.4 | 540 - 580 |
| Rhodamine B | 1.96 | 479 | 52.8 | 1 / 5 | 130.4 | 540 - 600 |
| Rhodamine B isothiocyanate | 2.03 | 536 | 86.5 | 1 / 7 | 116.6 | 540 - 600 |

[a)] Values determined by the shake flask method (according to OECD guideline for the testing of chemicals section 1: physical-chemical properties, test 107)
[b)] Values taken from Pubchem
PSA: polar surface area

**Table S2**. Transport study and quantification parameters of employed ß-blockers and antibiotics. Concentrations of ß-blockers and antibiotics initially added to the apical compartment of IM or mammalian comparator model-coated Transwell® filter inserts for permeability investigations, together with drug quantification parameters.

| Compound | Initial donor concentration (mM) | $\lambda_{max}$ (nm) | Binary solvent system (A:B) | Flow rate (ml $min^{-1}$) | Retention time (min) |
|---|---|---|---|---|---|
| Atenolol | 3.6 | 280 | 90:10 | 0.2 | 2.4 |
| Metoprolol | 16.4 | 280 | 70:30 | 0.4 | 1.1 |
| Timolol | 7.8 | 280 | 80:20 | 0.4 | 1.7 |
| Nadolol | 5.7 | 280 | 85:15 | 0.2 | 3.1 |
| Acebutolol | 0.89 | 235 | 70:30 | 0.2 | 2.2 |
| Alprenolol | 4 | 270 | 65:35 | 0.4 | 1.4 |
| Ciprofloxacin | 0.06 | 280 | 77:23 | 0.2 | 3.6 |
| Minocycline | 2.19 | 270 | 80:20 | 0.2 | 4.2 |

**Table S3**. Size and surface charge of bacteria- and mammal-specific liposomes. Z-average (nm) and surface charge (ζ-potential, mV) of POPE:POPG:CL (bacteria-specific) and PC (mammal-specific) liposomes. Values represent mean ± SEM from 4 batches, each measured in triplicate.

| **Liposome Formulation** | **z-average (nm)** | **ζ -potential (mV)** |
|---|---|---|
| **POPE:POPG:CL** | 413.5 ± 13.7 | -31.3 ± 1.4 |
| **PC** | 665.8 ± 30.4 | -4.9 ± 0.3 |

**Analysis of surface pressure isotherms**

The area occupied by phospholipid (PL) molecules in the bacterial PL mixed monolayer, at each surface pressure ($\pi$), were calculated according to the additivity rule as shown in equation S1:

$$[A_{TH}]_\pi = [X_{POPE}\, A_{POPE} + X_{POPG}\, A_{POPG} + X_{CL}\, A_{CL}]_\pi \quad \text{(S1)}$$

where $[A_{TH}]_\pi$ is the theoretical molecular area, X is the PL molar fraction and A is the PL molecular area in the pure PL monolayer.

The excess molecular areas $\Delta A^{EXC}$ were then calculated from the difference between the theoretical and experimental ($A_M$) molecular area values: $A_M$-$A_{TH}$. Negative deviations from the additivity rule indicate area condensation and may imply intermolecular accommodation and/or dehydration interactions between lipids in the mixed films; positive deviations from the additivity rule are the result of area expansion and would account for unfavorable interactions between the different components leading to poor mixing [1].
The free energy of mixing $\Delta G^M$ was calculated according to the Equation S2 [2]:

$$\Delta G^M = \Delta G^{EXC} + \Delta G^{IDEAL} \quad \text{(S2)}$$

where $\Delta G^{EXC}$ is the excess free energy (shown in equation S3) and $\Delta G^{IDEAL}$ the free energy of mixing (shown in equation S4) for an ideal mixture.

$$\Delta G^{EXC} = \int_0^\pi \Delta A^{EXC}\, d\pi \quad \text{(S3)}$$

$$\Delta G^{IDEAL} = RT\,(X_{POPE} \ln X_{POPE} + X_{POPG} \ln X_{POPG} + X_{CL} \ln X_{CL}) \quad \text{(S4)}$$

with R the universal gas constant and T the absolute temperature.

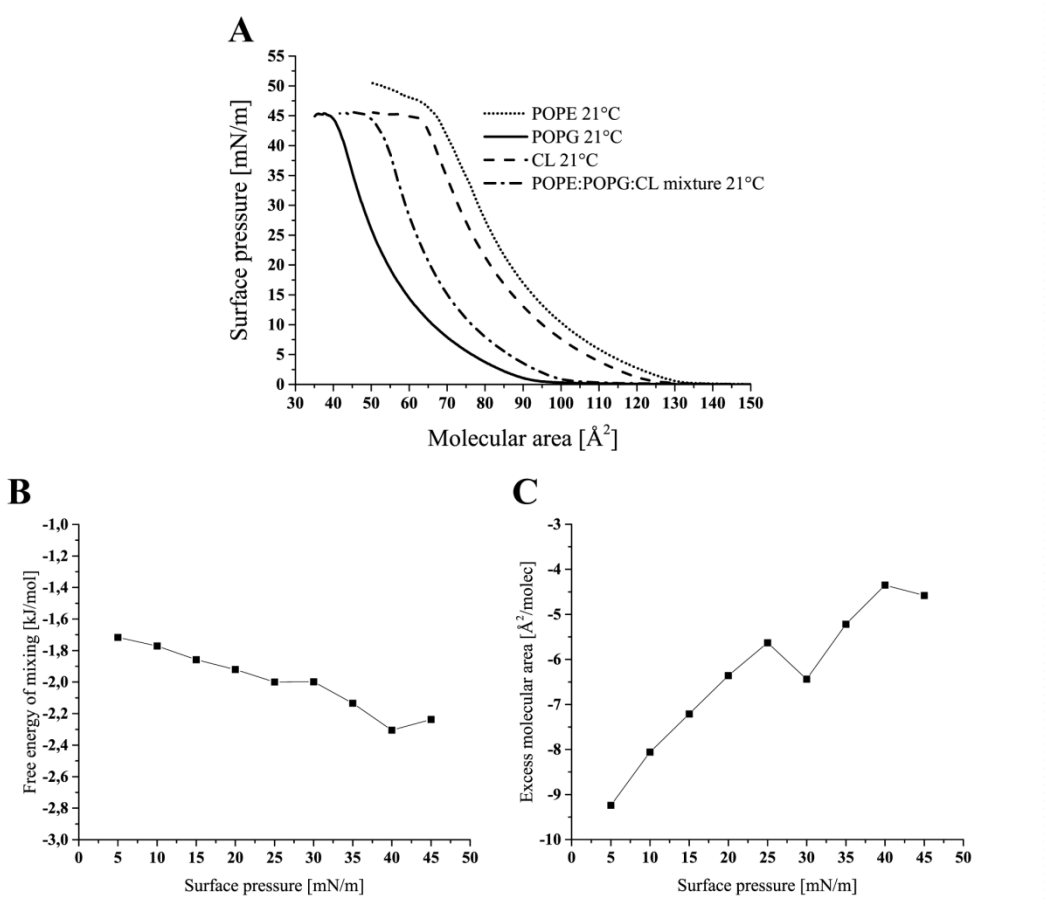


**Fig. S1.** Langmuir studies of pure bacteria-relevant PLs and physiological mixture. Comparison of the isotherms for the pure bacteria-relevant PLs and the inner membrane (IM) relevant mixture (A), together with the free energy of mixing ($\Delta G^{M}$) (B) and the excess molecular area ($\Delta A^{EXC}$) (C) with respect to surface pressure of the ternary PL blend. The occurrence of negative excess molecular area and free energies account for a good miscibility of the components of the ternary blend at all studied surface pressures, indicating an appropriate PL mixture.

**Electrical resistance measurements**

Simulated transport experiments were performed by exposing the IM model to Krebs-Ringer buffer (KRB; adjusted to pH 7.4). Resistance (R; Ω) was subsequently measured over 5 h, using a handheld chopstick electrode (STX-2) and an epithelial voltohmmeter (EVOM, World Precision Instruments, Berlin, Germany). Cell culture plates containing models coated onto Transwell® filter inserts were placed on an orbital shaker (IKA®-Werke GmbH and Co KG, Staufen, Germany) set at 150 rpm and kept in an incubator at 37 °C between the measurements. The electrical resistance (ER) provided by each model membrane at each measured time point was calculated via equation S5:

$$ER\ (\Omega * cm^2) = (R_{insert\ with\ model\ membrane} / R_{blank\ insert}) * 1.12\ cm^2 \quad (S5)$$

where 1.12 $cm^2$ represents the surface area of the Transwell® filter insert.

ER as such constitutes a parameter which provides an indication of barrier properties, with a resistance value of 400 Ω $cm^2$ often being considered as an accepted minimum value in live cell-based models [3,4].

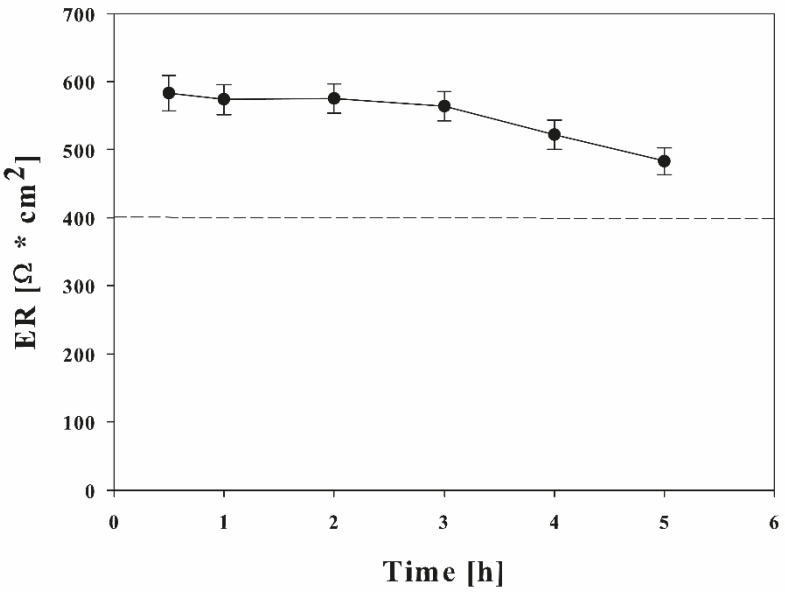


**Fig. S2.** Blank corrected ER values of the bacterial IM model. The high and relatively constant ER profile indicates sufficient model barrier properties. The dashed line indicates the accepted minimum ER level of live cell-based models such as Caco-2 cell monolayers, for reference. Values represent mean ± SEM; n=6 from 2 individual experiments.

**Optical topography evaluation of IM and mammalian comparator model surfaces**

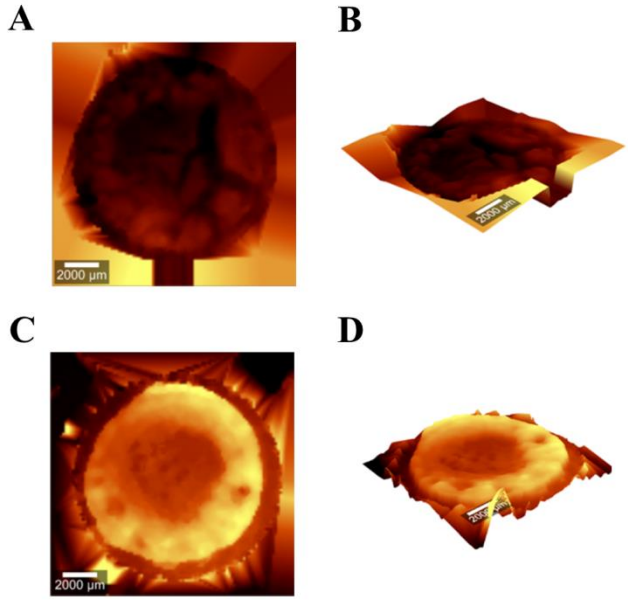


**Fig. S3.** Optical topography profiles of the overall model surfaces. Two- and three-dimensional optical topography profiles of the overall model surface area in case of the IM (A, B) as well as the mammalian comparator model (C, D).

## Cryogenic scanning electron microscopy (cryo-SEM) evaluation

Both IM and mammalian comparator models were frozen in liquid nitrogen to conserve the inner morphology followed by the preparation of cross-sections. Samples were further cryo-coated using a fracture coater (MED 020, Leica microsystems GmbH, Wetzlar, Germany) and subsequently imaged using a Quanta™ FEG 3 D SEM (FEI, Hillsboro, Oregon, USA).

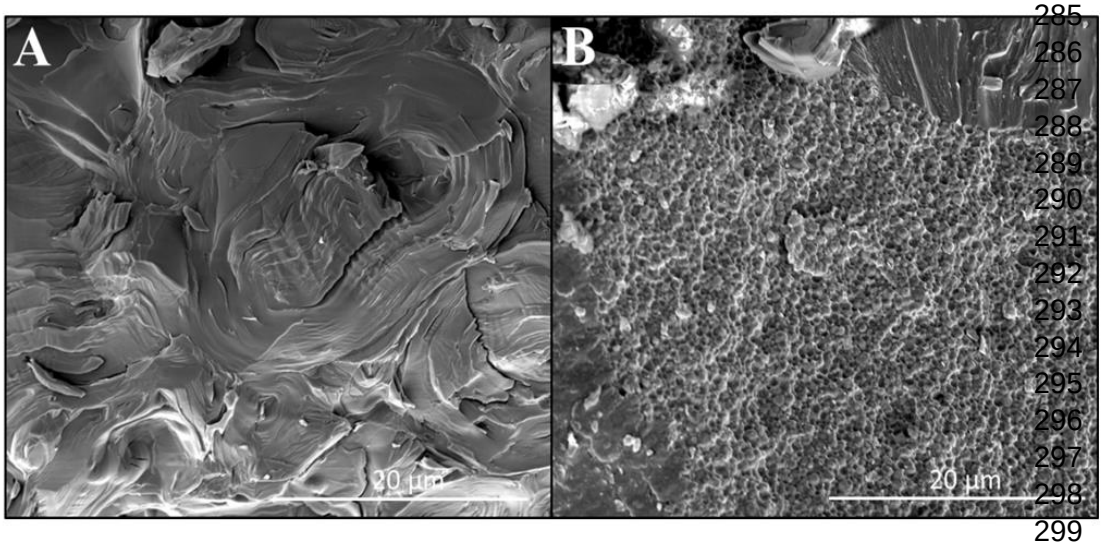


**Fig. S4.** Inner morphology assessment via cryo-SEM**.** Cryo-SEM cross section images of the bacterial IM (A) and mammalian comparator model (B) indicating differences in inner morphology as a result of the different lipid composition.

**Comparison of ß-blocker apparent permeability coefficient ($P_{app}$) values of the original phospholipid-vesicle based permeation assay (PVPA) and the mammalian comparator model (both prepared using PC as a mammal-relevant lipid)**

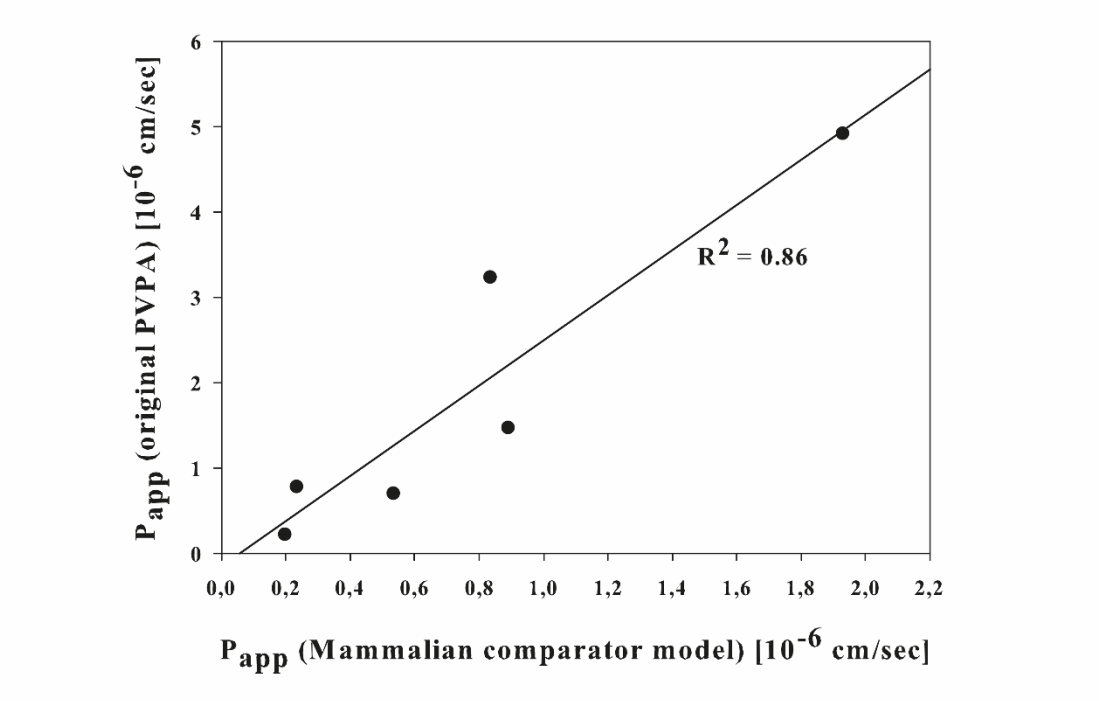


**Fig. S5.** Comparison of ß-blocker $P_{app}$ values in the current mammalian comparator model (abscissa) with corresponding $P_{app}$ values of the same compounds in the original human intestinal-mimicking PVPA (ordinate). A considerable correlation between $P_{app}$ data obtained in the two models is indicated, confirming that the current mammalian comparator model provides relevant and acceptable mammal-specific permeation information.

**Structures of fluorescently labeled muraymycin A-derived functionalized fatty acid and reference compound:**

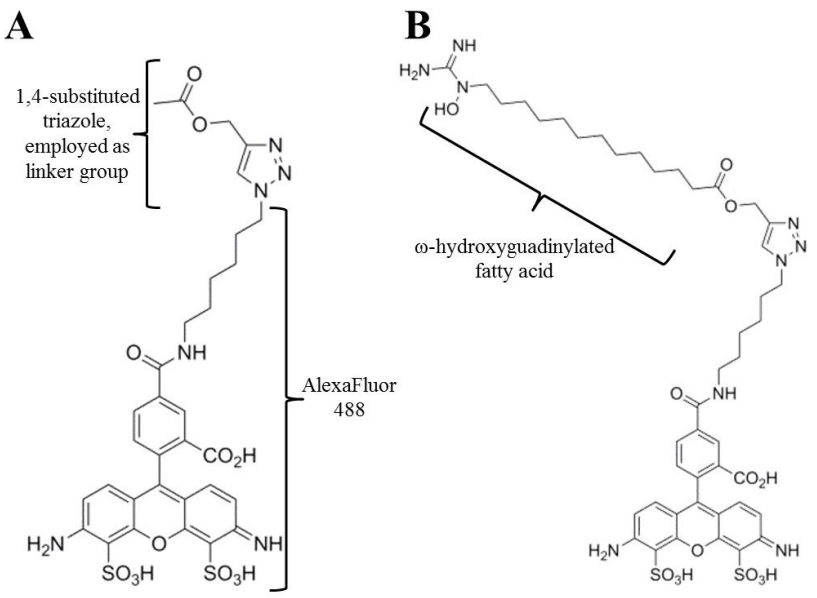


**Fig. S6.** Anti-infective derived structures utilized for permeability investigations.. Employed reference compound composed of the fluorescent label (AlexaFluor 488) and the linker group (A), as well as the fluorescently labeled ω-hydroxyguanidinylated fatty acid moiety, which were employed to demonstrate the validity of the IM model as well as emphasize its advantages in comparison to an existing *in vitro* bacterial permeation model. The model conjugate of the ω-hydroxyguanidinylated fatty acid has been conceived based on the structure of the naturally occurring nucleoside antibiotic muraymycin A1 [5].

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